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Short communication

Enzymatic determination of biogenic amines with transglutaminase

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Abstract

Tyramine, histamine, putrescine and cadaverine, the most common biogenic amines indicating the food quality, were studied in the transglutaminase-catalyzed reaction. Transglutaminase (protein-glutamine gamma-glutamyltransferase EC 2.3.2.13) catalyzes an acyl transfer reaction between a donor substrate and an acceptor substrate (e.g. biogenic amine) and forms a cross-linkage between substrates with a release of ammonia. The reaction can be monitored by measuring the ammonia produced in the reaction. The concentration of produced ammonia was found to be proportional to the concentration of biogenic amine and could hence be used to determination of biogenic amines in food matrixes.

Keywords: Tyramine; Histamine; Putrescine; Cadaverine; Transglutaminase; Detection

1. Introduction

Biogenic amines are organic bases which are found in plants, animals, humans and microorganisms. The occurrence of biogenic amines is related to bacterial growth and metabolism and they may be considered as indicators of spoilage for meat, fish, dairy products and wine [1,2]. Especially tyramine, putrescine and cadaverine have been reported to be useful indicators for estimating bacterial spoilage of meat [3] and elevated levels of histamine due to a metabolism of free histidines have been noted in fish spoilage [4]. These biogenic amines have been in interest in this work, studies of other biogenic amines can be found in the literature [3–6]. Large amounts of biogenic amines are formed due to the decarboxylation of amino acids by bacterial enzymes or alternatively due to the amination and transamination of aldehydes and ketones [2,4,7–9]. In food products the main route for formation of biogenic amines is the breakdown of amino acids catalyzed by microbial decarboxylases [2].

The determination of biogenic amines in food is important since they can be considered as indicators of food quality and also due to their effects on human health. Consumption of food containing high amounts of biogenic amines may have toxicological effects, such as nausea, sweating, headache and hyper- or hypotension [2,4]. Histamine is also known to be an inducer of allergic reactions and it also causes the so-called scombroid poisoning [4]. Normally the amines obtained from food are detoxified in the human metabolism by amine oxidases or by conjugation, but if the detoxification does not take place or is insufficient to cope with abnormally high intakes, the amine accumulates and causes the symptoms mentioned above [2,4].

Traditionally, biogenic amines have been determined with chromatographic methods such as gas, liquid, high-performance liquid chromatography, paper, thin layer, paper and capillary electrophoresis, which all often require laborious derivatization pre-treatment of samples and hence longer experiment times up to 2 h [3,5,10–13]. Furthermore the equipment required, e.g. high-performance liquid chromatograph or gas chromatograph, is expensive. The advantages are low detection limits and the possibility to separate bio-

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genic amines. Another approach can be radioimmunoassay or enzyme linked immunosorbent assay [12]. These methods do not need the sample pre-treatment and are fast, though they have modest specificity and it is difficult to obtain antibodies against putrescine.

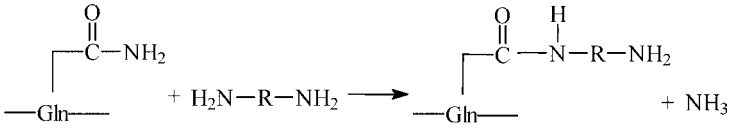
Enzymatic methods can also be used for the determination of biogenic amines. Biogenic amines can be converted to the corresponding aldehydes by amine oxidases, which are enzymes catalyzing fast oxidative deamination (reaction (1)) [7,13–16]. In this reaction hydrogen peroxide and ammonia are released. Hydrogen peroxide can in turn be detected as a coloured reaction product by adding a peroxidase enzyme and a suitable chromogen (reaction (2)).

$$R{-}CH_2{-}NH_2 + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{amine}} RCHO + NH_3 + H_2O_2 \quad (1)$$

$$H_2O_2 + \text{chromogen} \xrightarrow{\text{peroxidase}} \text{colour} + H_2O \quad (2)$$

The produced hydrogen peroxide can be detected by many ways e.g. by amperometric, chemiluminescence and spectrophotometric methods [13,16–21]. Several enzyme sensors based on amine oxidases have been constructed. Limitations in sensors are the long-term stability and intractability of catalytic membranes [21]. The substrate specificity might be a limitation for the individualization of the amine in the reaction since several amines can be used as substrates [13]. The sensor advantages are fastness, usability and the sufficient sensitivity, even though it is 10 or 20 times less than e.g. with high-pressure liquid chromatography.

Transglutaminase catalyses an acyl transfer reaction between the donor, the γ-carboxamide group of protein-bound glutamine (Gln) residue, and the acceptor, the amino group of primary amine, diamine or polyamine in peptides or proteins (reaction (3)) [22–24]. In proteins the 6-amino-groups of protein- and peptide-bound lysine residues act as acceptors, to give intra- and inter-molecular N^6-(5-glutamyl)-lysine cross-links. The amino groups of biogenic amines can function as acceptor groups, and also other amines such as ethylenediamine, hexamethylenediamine, spermine, spermidine and 3,3′-iminobispropylamine have been reported to function as acceptors. These polyamines function in vitro as acceptor substrates due to their protonated amine groups [22,23]. In the reaction a cross-linkage is formed and ammonia is released as a by-product. This ammonia can subsequently be measured with a spectrophotometric assay



Donor Acceptor (3)

Transglutaminase is widely used in various food applications e.g. to gelatinise food products, to improve the solubility, water-holding capacity or thermal stability of food proteins and to restructure food products in order to improve their flavour, nutritional value, appearance or texture [25]. However, the use of transglutaminase has not previously been reported for the determination of biogenic amines.

The aim of this work was to develop and evaluate an enzyme-aided reaction where no pre-treatments is required for the determination of the total concentration of tyramine, histamine, putrescine and cadaverine in food samples.

2. Materials and methods

2.1. Substrates and enzyme

Tyramine and histamine dihydrochloride were purchased from Sigma–Aldrich. Dihydrochloride salts of putrescine and cadaverine were purchased from Fluka and Sigma–Aldrich. Transglutaminase (EC 2.3.2.13, 1670 nkat/g) was purchased from Ajinomoto Co. Inc., Japan (Europe Sales GMBH, Hamburg, Germany). The donor substrates glutamine-glycine (Gln-Gly) and *N*-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) were purchased from Sigma–Aldrich. The donor substrate casein (nach Hammarsten) was obtained from Merck. The spectrophotometric ammonia assay was supplied by Boehringer Mannheim. A 100 mM Tris–HCl (pH 7.0) was used as a buffer in all experiments. The enzyme activity used throughout the work was based on the information provided by the enzyme suppliers.

2.2. Enzymatic reactions

In the acyl transfer reaction Tris–HCl buffer was pipetted to a test tube. The donor substrate, which was either Gln-Gly (20 mM, 0.5 ml), casein (1–10 mg/ml, 0.5–4 ml) or CBZ-Gln-Gly (0.5–4.0 mM, 0.5–2 ml) was added to the test tube. Amine was added to obtain a final concentration of 0.05–2.0 mM. The reaction was started by adding 0.25–0.75 ml transglutaminase (8.3–25.1 $\text{nkat/ml}_{\text{reaction mixture}}$). The final reaction volume was 5.0 ml with Gln-Gly and casein as the donor substrate and 2.5 ml with CBZ-Gln-Gly as the donor substrate. The reaction was monitored as a function of time by analyzing the produced ammonia using the kit assay, based on the reaction of ammonia with 2-oxoglutarate to L-glutamate in the presence of glutamate dehydrogenase and NADH as

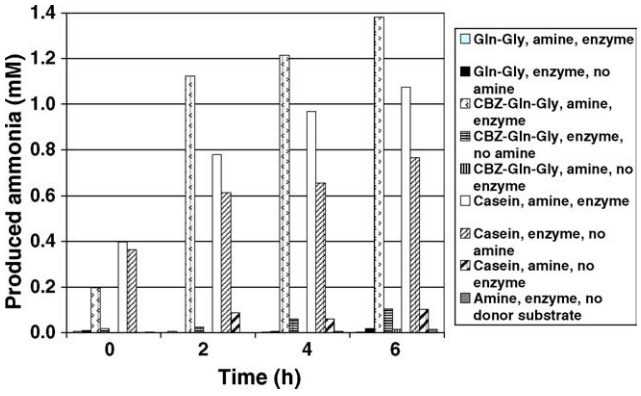


Fig. 1. The amount of produced ammonia in transglutaminase (16.7 nkat/$ml_{reaction\ mixture}$) catalyzed reaction with and without biogenic amine putrescine as acceptor substrate when reduced glutathione (2.0 mM), Gln-Gly (2.0 mM), CBZ-Gln-Gly (2.0 mM) and casein (5 g/l) were used as donor substrates with and without enzyme (pH 7.0, 25 °C, 0, 2, 4, 6 h).

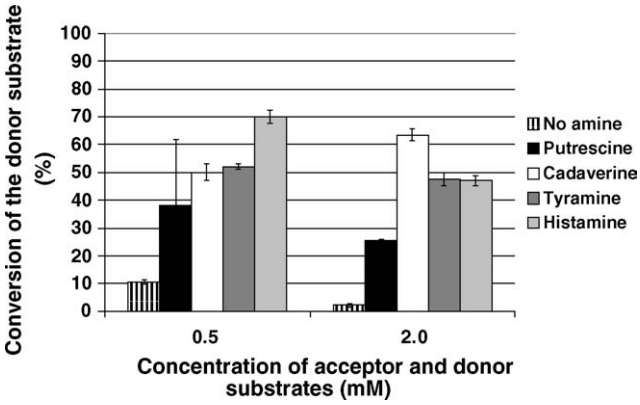


Fig. 2. The amount of produced ammonia in the conversion of the donor substrate *N*-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) by transglutaminase (8.3 nkat/$ml_{reaction\ mixture}$), reaction time 8 h. Concentrations of acceptor (biogenic amines) and donor (CBZ-Gln-Gly) substrates were equal (0.5 or 2.0 mM).

a cofactor. The absorbance of NAD at 340 nm with Hitachi 2000 spectrophotometer gave the amount of ammonia.

All determinations throughout the work were performed in duplicate except for the preliminary comparison of the different donor substrates.

3. Results and discussion

3.1. Comparison of different donor substrates

The suitabilities of different donor substrates, i.e. casein, glutamine-glycine (Gln-Gly) and *N*-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly), were first tested using putrescine as the acceptor substrate in the transglutaminase-catalyzed reaction (Fig. 1). By definition the natural acyl donor in transglutaminase reactions is protein-bound gamma glutamine (IUBMB Enzyme Nomenclature) thus although Gln-Gly contains a glutamine residue, it could not act as donor substrate for transglutaminase and practically no ammonia was produced. Most probably Gln-Gly did not have the amino acid groups positioned in a proper way for transglutaminase to identify their glutamine residues. As previously reported [26,27], casein was found to be a good substrate for transglutaminase. Even without putrescine substantial amounts of NH_3 were produced from casein, indicating that cross-linking between or within the casein molecules occurred. The added amine had a relatively low contribution to the formation of cross-links in the presence of casein and thus casein cannot be used as a donor substrate for transglutaminase in analytical applications.

N-Carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) has been reported to be a good donor substrate for microbial transglutaminase [24]. In this work it was also found to be a very suitable donor for the transglutaminase reaction. When the formed cross-linkages between biogenic amines and donor substrate were calculated on the basis of the first tests it was found that the amount of cross-links was 0.69 $mmol/mmol_{CBZ\text{-}Gln\text{-}Gly}$ with 2.0 mM putrescine and 0.05 $mmol/mmol_{CBZ\text{-}Gln\text{-}Gly}$ without putrescine showing though a clear difference in the reaction with and without putrescine. Since practically very low reaction was obtained without any amine, CBZ-Gln-Gly appeared to be a promising donor substrate for analytical application of transglutaminase. In order to confirm the suitability of CBZ-Gln-Gly, the enzymatic activity of transglutaminase towards different biogenic amines was studied using CBZ-Gln-Gly as a donor substrate. With donor and acceptor concentrations of 0.5 and 2.0 mM, clear reactions with 25–70% conversion of CBZ-Gln-Gly were observed for all studied amines (Fig. 2). Without the amine the conversion of CBZ-Gln-Gly was 2–10%.

3.2. Development of the determination method using CBZ-Gln-Gly as donor substrate

The determination method was further investigated and developed using CBZ-Gln-Gly as the donor substrate (concentration 2 mM unless otherwise stated). The reaction was also monitored as a function of reaction time. The concentration of produced ammonia increased rapidly during the first 2 h of the reaction (Fig. 3). After 8 h the production of ammonia almost ceased in the conditions used (donor substrate 2.0 mM, transglutaminase 8.35 nkat/$ml_{reaction\ mixture}$). Cadaverine was the best acceptor substrate for transglutaminase and thus the highest concentration of ammonia was produced from cadaverine. With tyramine and histamine the production of ammonia was about the same, whereas putrescine had the lowest response to the reaction even though the structure of putrescine is similar to that of cadaverine with a difference of one $-CH_2$ group in the chain. However, amine acceptors have reported to cause substrate inhibition at least for some transglutaminases [28].

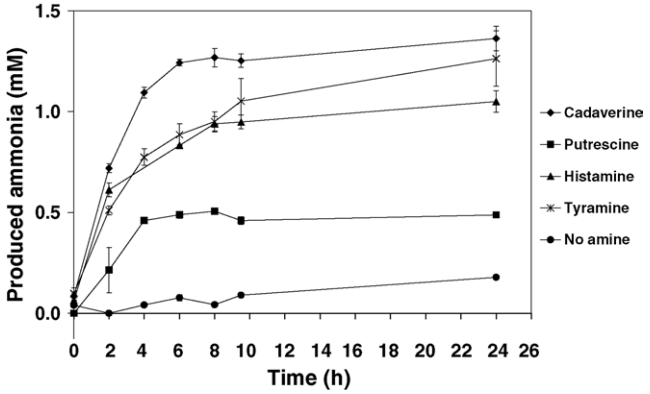


Fig. 3. The amount of produced ammonia in transglutaminase (8.3 nkat/$ml_{reaction\ mixture}$) catalyzed reaction as a function of time in a reaction with *N*-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) (2.0 mM) as donor substrate and biogenic amines (2.0 mM) as acceptor substrates (pH 7.0, RT).

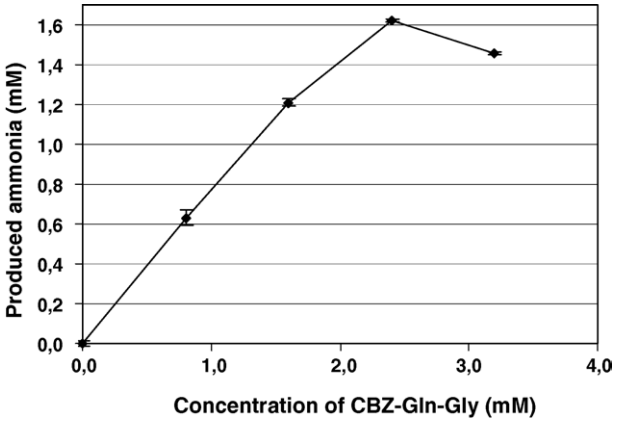


Fig. 4. The amount of produced ammonia in transglutaminase (8.3 nkat/$ml_{reaction\ mixture}$) catalyzed reaction with different concentrations of *N*-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) as donor substrate and with cadaverine (2.0 mM) as acceptor substrate, reaction time 6 h.

The effect of the concentration of the donor substrate CBZ-Gln-Gly was further studied using 2.0 mM putrescine or cadaverine as acceptor substrate. Surprisingly, it was found that with a concentration of 3.2 mM CBZ-Gln-Gly, less ammonia was produced than with a concentration of 2.4 mM (Fig. 4). Apparently excess CBZ-Gln-Gly causes end product inhibition or substrate inhibition [29]. On the basis of this experiment a final concentration of 2.4 mM CBZ-Gln-Gly was selected for further experiments unless otherwise specified.

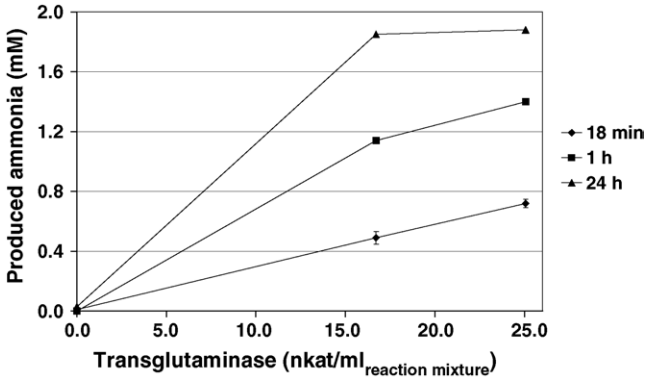


Fig. 5. The effect of the transglutaminase dosage on the amount of produced ammonia (mM) in transglutaminase-catalyzed reaction. Cadaverine (2.0 mM) was used as acceptor substrate and *N*-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) (2.4 mM) as donor substrate.

The effect of transglutaminase dosage was also elucidated in order to determine whether the reaction could proceed faster and whether higher amounts of produced ammonia could be attained. The amount of produced ammonia was 0.49 mM with a transglutaminase dosage of 16.7 nkat/$ml_{reaction\ mixture}$ and 2.0 mM cadaverine as the acceptor substrate after 18 min of reaction corresponding to 27 nmol/ml min whereas with a 1.5-fold dose of transglutaminase the amount of ammonia produced was 1.5-fold ammonia (Fig. 5). The solubility of the commercial transglutaminase preparation containing e.g. dextrins was a limiting factor in the optimization of the amount of transglutaminase, it was not possible to prepare homogenous transglutaminase solution with high enzyme concentrations.

The correlation of the produced ammonia with the concentration of biogenic amines was also investigated by plotting the amount of ammonia against the concentration of biogenic amines. The resulting graphs were found to be linear from 0.05 to 2.0 mM, and good correlation coefficients between 0.985 and 0.995 were obtained (Table 1, Fig. 6). The correlation was very similar for all the amines studied. In this experiment the highest concentrations of ammonia were again produced from cadaverine. The amounts of produced ammonia were very similar when putrescine and histamine were used as substrates for transglutaminase. When the conversion rate was calculated from the linear graphs it was found that at lower concentrations of biogenic amines the conversion rate was better e.g. the conversion rate of 0.5 mM cadaverine was 99% but with 2.0 mM cadaverine the conversion rate was 78%. For other amines

Table 1
Analytical data of biogenic amines in the transglutaminase-catalyzed reaction in the presence of *N*-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) (2.4 mM) and transglutaminase (25.1 nkat/$ml_{reaction\ mixture}$) (pH 7.0, 25 °C, 2 h)

	Putrescine	Cadaverine	Histamine	Tyramine
Linearity range (mM)	0.05–2	0.1–2	0.05–2	0.05–1
Slope (ΔmM/ΔmM)	0.701 ± 0.009	0.754 ± 0.031	0.703 ± 0.006	0.689 ± 0.007
Correlation coefficient	0.9954	0.9918	0.9946	0.9927
Lower detection limit (mM)	0.07	0.10	0.07	0.06

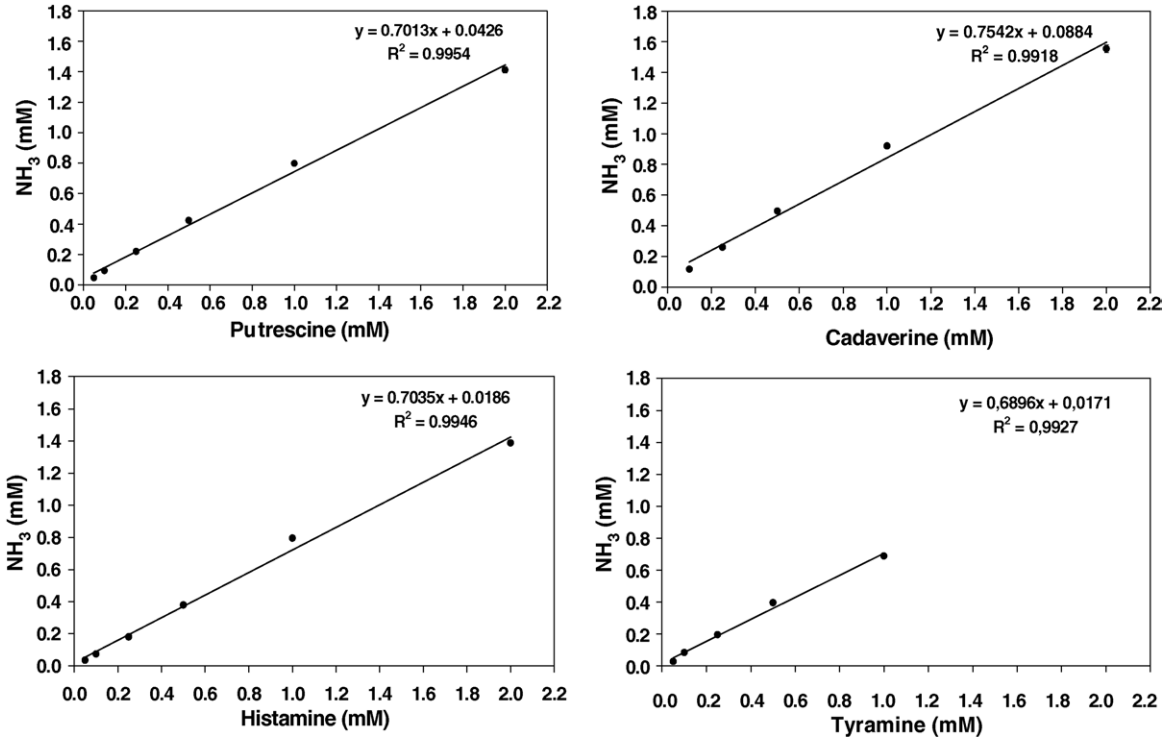


Fig. 6. The concentration of ammonia vs. concentration of the biogenic amines in transglutaminase catalyzed reaction in the presence of CBZ-Gln-Gly (2.4 mM) and transglutaminase (25.1 nkat/ml$_{\text{reaction mixture}}$) (pH 7.0, 25 °C, 2 h).

the conversion was 85–57% within the concentration range 0.5–2.0 mM.

The K_m values were studied in order to determine the sensitivity of transglutaminase towards biogenic amines, and to confirm the concentration range of biogenic amines in which this reaction can be used. The K_m values of transglutaminase for putrescine, cadaverine, tyramine and histamine were 6.0, 3.4, 3.9 and 5.9 mM. These values indicate that the transglutaminase-catalyzed reaction could be used e.g. for determination of these amines in spoiled food products. It has been reported that fresh meat contains 0.04 mM histamine, 0.09 mM putrescine and 0.1 mM cadaverine [4], whereas typical concentrations of these amines in spoiled meat vary up to 0.4 mM [1]. It has been recommended that the histamine concentration should not exceed 0.9 or 1.8 mM (100 or 200 mg/kg) [4,13,30,31]. The threshold histamine concentration for toxicological effects has been estimated to be 4.5–9.0 mM (500–1000 mg/kg) [30,32]. Corresponding values for tyramine are 0.7–5.4 mM (100–800 mg/kg) [4,31].

4. Conclusions

Transglutaminase is a bulk enzyme, which can be produced inexpensively. The reaction is simple and rapid (18 min) to perform, and could be used for continues on-line measurements. It does not require any pre-treatment or additional reactants (e.g. oxygen) in addition to the acceptor and donor substrate for the determination of total levels of amines. This is a clear advantage in comparison to e.g. of expensive amine oxidases, which have been used for the determination of biogenic amines or compared to the traditional methods, which are costive and require normally almost an hour for derivatization plus the analyze time. Transglutaminase catalyzes the cross-linking of several molecules and has broad specificity. In this work potential side reactions and interferences caused by other components in spiked samples were not yet studied. Further studies are needed to optimize the reaction. In future transglutaminase could be used as the amine sensitive component to detect ammonium by e.g. ion selective electrodes.

The transglutaminase-catalyzed reaction seems a promising tool for the determination of total levels of biogenic amines.

Acknowledgment

This work is part of the VTT Biotechnology top competence area entitled “Active and smart consumer packaging”.

References

[1] T. Hernández-Jover, M. Izquierdo-Pulido, T. Veciana-Nogués, A. Mariné-Font, C. Vidal-Carou, J. Agric. Food Chem. 45 (1997) 2098.
[2] R. Maijala, Formation of Biogenic Amines in Dry Sausages, with Special Reference to Raw Materials, Lactic Acid Bacteria, pH Decrease, Temperature and Time, Yliopistopaino, Helsinki, 1994, ISBN 952-90-5687-7.

[3] M. Rokka, S. Eerola, M. Smolander, H.-H. Alatomi, R. Ahvenainen, Food Contr. 15 (2004) 601.
[4] A. Halász, Á. Baráth, L. Simon-Sarkadi, W. Holzapfel, Trends Food Sci. Technol. 5 (1994) 42.
[5] M.R. Alberto, M.E. Arena, M.C. Manca de Nadra, Food Contr. 13 (2002) 125.
[6] Y.-H. Tsai, H.-F. Kung, T.-M. Lee, H.-C. Chen, S.-S. Chou, C.-I. Wei, D.-F. Hwang, Food Contr. 16 (2005) 579.
[7] J. Gouygou, C. Martin, C. Sinquin, Oceanis 15 (1989) 599.
[8] S. Bardócz, Trends Food Sci. Technol. 6 (1995) 341.
[9] A. Bouchereau, P. Guénot, F. Larher, J. Chromatogr. B 747 (2000) 49.
[10] E. Pyra, S. Wawrzycki, B. Modzelewska-Banachiewicz, Chromatogr. Suppl. 51S (2000) 414.
[11] R. Romero, M. Bagur, M. Sánchez-Viñas, D. Gázquez, Chromatographia 51 (2000) 404.
[12] D. Teti, M. Visalli, H. McNair, J. Chromatogr. B 781 (2002) 107.
[13] J. Lange, C. Wittmann, Anal. Bioanal. Chem. 372 (2002) 276.
[14] R.E. Childs, W.G. Bardsley, Biochem. J. 145 (1975) 93.
[15] P.A. Lerke, M.H. Porcuna, H.B. Chin, J. Food Sci. 48 (1983) 155.
[16] M.J.E. Hewlins, T.C. O'Dowd, R.H. Davis, P.J. Winterburn, PCT-application WO8907152 (1989).
[17] K.B. Male, P. Bouvrette, J.H.T. Luong, B.F. Gibbs, J. Food Sci. 61 (1996) 1012.
[18] R. Draisci, G. Volpe, L. Lucentini, A. Cecilia, R. Federico, G. Palleschi, Food Chem. 62 (1998) 225.
[19] M. Wimmerovà, L. Macholàn, Biosens. Bioelectron. 14 (1999) 695.
[20] I. Frébort, L. Skoupá, P. Pec, Food Contr. 11 (2000) 13.
[21] H. Okuma, W. Okazaki, R. Usami, K. Horikoshi, Anal. Chim. Acta 411 (2000) 37.
[22] J. Schrode, J.E. Folk, J. Biol. Chem. 254 (1978) 4847.
[23] J.E. Folk, M.H. Park, S.I. Chung, J. Schrode, E.P. Lester, H.L. Cooper, J. Biol. Chem. 255 (1980) 4695.
[24] H. Ando, M. Adachi, K. Umeda, A. Matsuura, M. Nonaka, R. Uchio, H. Tanaka, M. Motoki, Agric. Biol. Chem. 54 (1989) 2613.
[25] G. Wijngaards, Y. Zhu, J.M. Maagd, J. Bol, Med. Fac. Labouww. Univ. Gent. 62/4a (1997) 1381.
[26] G.R. Lilley, M. Griffin, P.L.R. Bonner, J. Biochem. Biophys. Meth. 44 (1997) 41.
[27] J. Choi, G. Ho Choi, K. Mee Woo, S.C. Park, Seoul J. Med. 33 (1992) 167.
[28] J.D. Klein, E. Guzman, G.D. Kuehn, J. Bacteriol. 174 (1992) 2599.
[29] A. Madi, M. Punyiczki, M. di Rao, M. Piacentini, L. Fesus, Eur. J. Biochem. 253 (1998) 583.
[30] M. Hall, D.B. Eldridge, R.D. Saunders, D.L. Fairclough, R.C. Bateman Jr., Food Biotechnol. 9 (1995) 39.
[31] E. Petäjä, S. Eerola, P. Petäjä, Eur. Food Res. Technol. 210 (2000) 280.
[32] G.C. Chemnitius, U. Bilitewski, Sens. Actuators B 32 (1996) 107.