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The Cytotoxic and Genotoxic Effects of Conjugated Trans-2-Nonenal (T2N), an Off-Flavor Compound in Beer and Heat Processed food Arising from Lipid Oxidation

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A b s t r a c t

This study investigates the toxic effect of E(2)nonenal (trans-2-nonenal, T2N) and its conjugate with horse muscle myoglobin (Mb) tested on murine cell line L_{929} and human cell line A_{549}, as well as the genotoxic effect of these compounds assayed by measuring of micronuclei in human cells K_{562}. It is an aldehyde, which is occurring as the substance responsible for an off flavour in aged beers, but originates also from lipid oxidation in heat processed food. T2N is an aldehyde formed from linoleic acid as a secondary oxidation product. The modification of Mb with T2N was analyzed with the use of SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and electrospray ionization mass spectrometry (ESI-MS). Results from SDS-PAGE suggest that T2N substitutes Mb and additionally causes cross-linking with polymerization of Mb resulting in an insoluble fraction. The ESI-MS spectrum of the soluble fraction used in the toxicity tests, demonstrated that conjugation of T2N with Mb yielded Mb adducts with one residue of trans-2-nonenal per myoglobin molecule as the major fraction and adducts with different numbers of T2N molecules as minor fractions. In the cytotoxicity assay the T2N and its Mb conjugate causes 50 % destruction of cells at the concentration 95–125 µg/ml and 200 µg/ml respectively, when L_{929} and A_{549} cell lines were used, whereas Mb control tested up to 2000 mg/ml was without any cytotoxic effect. In genotoxicity *in vitro* assay we have observed that the T2N and its Mb conjugate expressed the genotoxicity. The number of micronuclei in human K_{562} cells reached 26 ± 2.16 promille (MN/1000 cells), comparing to 62 ± 8.64 MN/1000 cells for the reference free T2N, whereas a control value was 10.33 ± 1.25 MN/1000 cells. The studied compounds expressed also the apoptotic effect in K_{562} cells as the number of apoptotic cells increased to 44.67 ± 4.92 promille for T2N-Mb, comparing to 168.67 ± 37.28 promille for free T2N, whereas a control value was 30.33 ± 1.36 promille for Mb. In these assays the T2N-Mb conjugate is several times more toxic in relation to control protein. Results indicate that T2N adducts with protein are potent to induce various cytotoxic and apoptotic effects when assayed *in vitro* tests. It suggests that higher level of such aldehyde might create in organism severe potential of toxicity.

K e y w o r d s: cytotoxicity, genotoxicity, apoptosis, trans-2-nonenal, T2N, beer, malt, ESI-Mass Spectrometry, Mb-T2N adducts, myoglobin

Introduction

Food chemists have for a long time known that autoxidation of fats and oils, in particular those rich in polyunsaturated fatty acids (PUFA), is accompanied by the formation of aldehydes. The lipid peroxidation is connected with the formation of undesirable taste and flavour of cereals and other foods. In most cereals food lipid peroxidation causes reduction of quality and shelf life of the products (Hilderbrand, 1992). Beer is one of the food items whose flavour is deteriorated due to lipid oxidation during beer production.

Abbreviations: Mb, myoglobin; T2N, trans-2-nonenal; T2N-Mb, trans-2-nonenal myoglobin conjugate; ESI-MS, Electrospray Ionisation-Mass Spectrometry; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; LOX, lipoxygenase; 4-HNE, 4-hydroxynonenal.

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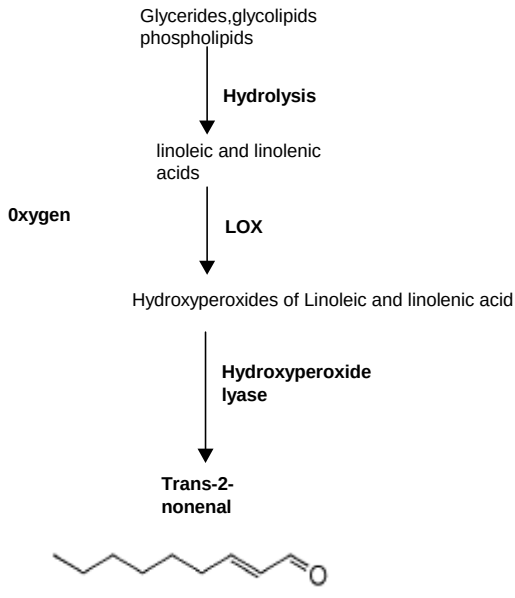

Figure 1. Enzymatic formation of trans-2-nonenal

Oxidation of lipids starts already during grain germination and is initiated by the action of lipoxygenases. Lipoxygenase-1 (LOX-1) together with hydroperoxide lyase contributes to the production of an off-flavour aldehyde trans-2-nonenal (T2N) (Fig. 1). This is an oxidation product from linoleic acid, the major fatty acid of the barley grain lipid.

This aldehyde is responsible for an off-flavor that has been described as stale, paper-like or astringent. According to Drost *et al.* (1990), enzymatic oxidation of unsaturated fatty acids during mashing is the second most important source for the formation of stale flavor aldehydes, beside the germination step. T2N like 4-hydroxy-nonenal (4-HNE) is a very reactive aldehyde and forms stable protein adducts. Among the many different aldehydes, which can be formed during lipid peroxidation, were malonaldehyde (MDA) and 4-hydroxyalke nals and in particular 4-HNE and 4-hydroxyhexanal (4-HH) have been most intensively studied. The most cytotoxic aldehyde is 4-HNE (Esterbauer *et al*., 1991), which has been found in many auto-oxidized food items, such as oil samples collected from the outbreak of the toxic oil syndrome in Spain 1981 (Esterbauer *et al*., 1991). The presence of 4-HNE has been documented in beef and pork at concentrations of 14–150 and 1–152 nmoles/g, respectively (Sakari *et al*., 1995).

The objective of this paper is to demonstrate that the off-flavor compound T2N, which can be present in beer in free or adducted form is cytotoxic. Since the T2N adduct usually are tasteless, they can be consumed without being noticed. In this context we discuss the need to develop a procedure for quantification of T2N and its adducts in raw materials used in the brewing process as well as to identify the type of adducts and their toxicity.

Experimental

Materials and Methods

Materials. E-2-nonenal (T2N) was purchased from Aldrich Chemical Co (Milwaukee, W, USA). Murine (C3H/An strain) connective tissue cell line L_{929}, human lung carcinoma cell line A_{549} (ATCC CCL-185) and human chronic myelogenous leukaemia cells K_{562} were from the stock collection of the Institute of Immunology and Experimental Therapy. Horse muscle myoglobin was from Sigma, Chemical Co. (St Louis, Mo, USA). All other reagents were pro-analysis.

Preparation of T2N-Mb adducts. Ethanol solvent (0.5 ml) was carefully removed at room temperature with a gentle stream of nitrogen from 3.85 mmoles of T2N solution and the sample was immediately dissolved in 0.5 ml of PBS, pH 7.3. Then 3 mg of myoglobin was added to this solution and the reaction mixture was incubated for 2 h at 37°C. Then the material was lyophilized to remove any unreacted T2N. The lyophilized sample was dissolved in 300 µl of water. A small part of the not dissolved material was separated by centrifugation at 10000 rpm at 4°C for 15 min. in an Eppendorf centrifuge. The soluble fraction was analyzed in ESI-MS and tested for cytotoxicity. Both fractions were analyzed by SDS-PAGE. Before the ESI-MS analysis 25 volumes of 2 M HCl in acetone were added under stirring for 20 minutes to the T2N-Mb conjugate in order to obtain heme-free protein (Faustman *et al*., 1999). Precipitated material was isolated by centrifugation, dissolved in 500 µl water and dialyzed against water *prior* to the analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was used to monitor the polymerization of the modified proteins. The samples (1 mg/ml) of Mb, and T2N-Mb were treated with SDS sample buffer containing β-mercaptoethanol. After electrophoresis, gels were stained with Coomassie Brilliant Blue. Protein standards (Amersham-Pharmacia, Sweden) ranging from 14 to 94 kDa were used. The SDS-PAGE was performed on 4% stacking gel and 15% running gel (Laemmli, 1970).

Cytotoxicity assay. Cytotoxicity of the compounds was determined on murine (C3H/An strain) connective tissue cell line L_{929} and human lung carcinoma cell line A_{549} (ATCC CCL-185). The cell monolayers were trypsinized, then suspensions prepared of 2×10^5 cells/ml of Eagle's minimum essential medium (EMEM) or Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% calf serum (CS). The above media were mixed with various doses of the compounds, seeded in the plastic 96-well micro plates, and incubated at 37°C for 48 h in the presence of 5% CO_2. CD_{50} was the minimal concentration of the compound, which caused approximately 50 % destruction of the cell culture, and was determined by microscopic examination of the cells (Piasecki *et al*., 1992).

Genotoxicity and apoptosis assays. Human chronic myelogenous leukaemia cells K_{562} (2×10^5/ml) were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal serum, antibiotics and detected reagents. The cultures were incubated for 24 hours after treatment with tested agents at 37°C and 5% CO_2. Cells were then collected, washed with PBS and slides for

microscopy studies were prepared. Air-dried preparations were fixed for 20 min. in a cold solution of 1% glutaraldehyde in 1/15 M phosphate buffer (pH = 7.5). Slides were then rinsed with distilled water and stained according to Feulgen method (Abend *et al*., 1995). Genetic alterations in cells exposed to irradiation as well as in controls (unexposed cells) were estimated as the frequency of cells with micronuclei and of cells showing signs of chromatin condensation characteristic for apoptotic processes. Microscopic preparations were analyzed using criteria described by Abend *et al*. (1995). Micronuclei originate from broken acentric chromosome fragments or from whole chromosomes that were not incorporated during cell division into nuclei of daughter cells. Both micronuclei appearance and apoptosis are genetic events that lead to cell death (Abend *et al*., 1995).

Electrospray Ionization Mass Spectrometry (ESI-MS). ESI-MS analyses were performed on a TSQ700 Finnigan MAT instrument (San Jose, CA, USA) equipped with a standard Finnigan ionization and electrospray probe. Data acquisition and analysis were performed on a DEC 3000 alpha workstation. Samples (~1 mg/ml) were diluted 1:5 with a mixture composed of MeOH/H_2O/acetic acid (1:1:0.05) and infused with nitrogen into the instrument at 2 µl/min. Spectral deconvolution was done with the Finnigan Bioworks software.

Results and Discussion

No studies have yet been published regarding the cytotoxicity of trans-2-nonenal (T2N) adducts, despite extensive literature on the occurrence of T2N during different steps of beer brewing. Beer flavor stability depends on: barley variety, malt, worth quality, including cultivation conditions of barley, malting, kilning, mashing and boiling conditions. During malt kilning and mashing-in, step-wise enzymatic degradation of lipids and fatty acids is continued and leads to the formation of stale flavor (off-flavor) substances such as T2N (Drost, 1990). During storage at room temperature, beer often develops cardboard-like stale flavor caused by the aldehyde trans-2-nonenal, which already at 0.1 ppb of T2N gives a marked off-flavor. This substance is an indicator substance to predict the long-term shelf-life of beers. Free T2N, like other aldehydes, is very reactive and due to its reactivity T2N is mainly present in the form of adducts, which can be transported from malt to beer. Free T2N is a volatile substance while the laboratory made adducts are water-soluble, not volatile and flavorless substances.

To study and compare the cytotoxicity of T2N with its adducts, a model conjugate T2N-Mb was made. We realize that Mb or proteins like Mb may not be present in malt and latter in beer, but it is useful to have a well characterized model to begin studies of adducts formation in a complex media which is for example during wort production. Since conditions are varying during the beer production and adducts with properties shown by T2N-Mb could be also present in the real samples, the conjugation of myoglobin with T2N was made under gentle conditions, namely at 37°C for 2h in PBS at pH 7.3. The mild temperature (37ºC) should simulate the temperature during mashing in, a step in wort production. Such temperature is one of the alternative temperatures used for testing so called trans-2-nonenal potential (Drost *et al*., 1990).

Similar to earlier observations made at the preparation of 4-HNE-myoglobin adducts (Lynch and Faustman, 2000), there was no significant loss of this aldehyde during the incubation with myoglobin. The obtained product was a mixture of free and modified myoglobin, which after lyophilization was partially soluble. The insoluble fraction yielded 17% of the total used for the reaction. The soluble fraction was characterized by SDS-PAGE and ESI-MS and used for the cytotoxicity studies.

The SDS-PAGE pattern indicates that the insoluble fraction (Fig. 2, lane 2) contains a mixture of cross-linked protein with molecular masses ranging from 35 kDa to above 94 kDa. The molecular mass of each band could be calculated from the Mw standards. Approximately 50% of the conjugate is represented by Mb substituted to different degrees by T2N. These data suggest that part of the T2N breaks down and cross-links protein molecules. Peroxidation has been suggested as one of the reasons for myoglobin cross-linking (Guillen-Sans and Guzman-Chozas, 1998). The pattern seen in lane 2 (Fig. 2) suggests that part of T2N has degraded to malonyl dialdehyde (MDA) one of the final products in lipid peroxidation, and cross-linked myoglobin into complexes containing a few molecules of myoglobin.

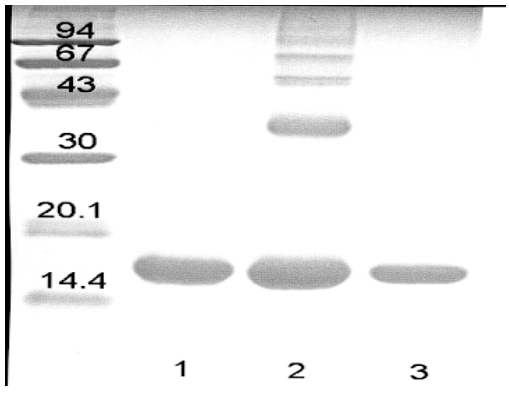

Fig. 2. SDS – polyacrylamide gel electrophoresis of T2N-Mb soluble fraction (1), T2N-Mb insoluble fraction (2) and original Mb (3). Each lane was loaded with 5 µg of material. The gel was developed with Coomassie Brilliant Blue stain.

The soluble products fraction subjected to the SDS-PAGE (Fig. 2, lane 1) corresponds to bands with only slightly changed Mw comparing to the Mb standard. Therefore, mass spectrometry (ESI-MS) was used to analyze the composition

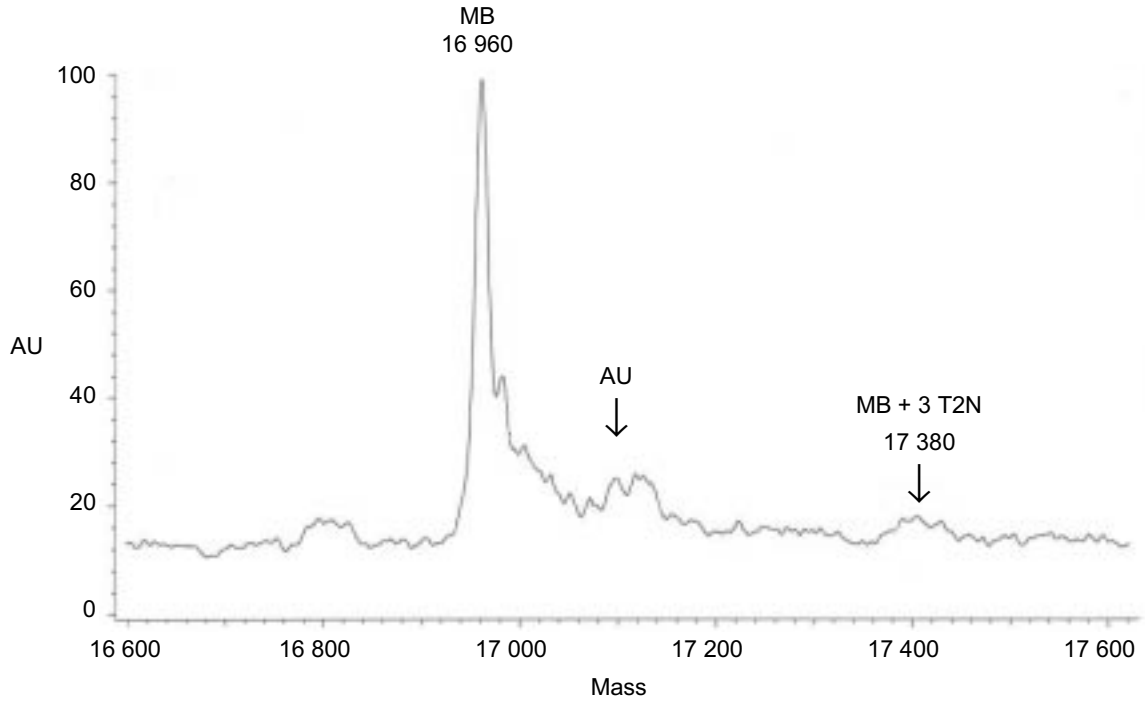

Figure 3. Electrospray-ionization mass spectrum of T2N-Mb conjugate

of the Mb-T2N adducts in this fraction (Fig. 3). The deconvoluted ESI-MS spectrum demonstrates that the soluble fraction comprised Mb-adducts with predominantly one and three residues of trans-2-nonenal per myoglobin molecule as the major peaks. In addition, a number of non-identified components are seen in the mass spectrogram. The peak at mass 16 960 is equivalent to horse muscle myoglobin without heme. The peak with a mass of 17 100 was 140 Da greater than that of myoglobin, a mass difference corresponding to the molecular mass of T2N. The intensities were decreasing of the series of the minor peaks representing the adduction of additional T2N molecules to Mb differing with multiples of 140 Da, although the series could be traced up to four T2N residues. By integration of the peaks it could be seen that approximately 40% of the Mb molecules contain T2N. These results show that T2N produced by lipid oxidation reacts with Mb. Similar results have been obtained by Faustman *et al.* (1999) who studied myoglobin adducts with 4-HNE. The aminoacids of myoglobin that can be adducted with T2N are: lysine, histidine, and cysteine, as was shown for 4-HNE adduction with myoglobin (Faustman *et al*., 1999). Our studies are the first studies with T2N-adduct formation. We realize that more systematic studies are required such as tryptic digestion and MS-based proteomic to elucidate which residues were modified.

The purpose of this study was to see if T2N-adducts can be formed with the model protein, Mb, and if so, if they are cytotoxic. We have focused on the soluble fraction and identified the adducts by ESI-MS (Fig. 3) and tested their cytotoxicity and genotoxicity (Tables I and II). Free T2N and Mb-T2N present in the soluble fraction, were found cytotoxic when tested on murine cells and on the human lung carcinoma cell line. T2N

Table I

Cytotoxicity of trans-2-nonenal (T2N) and its Mb adduct towards two types of cell lines. L_{929}, murine c., A_{549}, human lung adenocarcinoma., nt* tested up to 2000 μg/ml without any cytotoxic effect. The results are already expressed as the a mean obtained from tripilicate samples not exceeding ± 6%

Compound tested	Cytotoxicity CD_{50} (μg/ml) Cell line	
	L_{929}	A_{549}
1. E-2-nonenal	94	125
2. E-2-nonenal-Myoglobin conjugate	200	200
3. Myoglobin	nt*	nt*

Table II
Genotoxicity of trans-2-nonenal (T2N) and its Mb adduct towards cell line K_{562}, human chronic myelogenous leukaemia cells. The results are already expressed as the a mean obtained from tripilicate samples with their standard deviations

Compound tested	Concentration in culture [μg/ml]	Frequency of occurring cells [‰]			
		with micronuclei		apoptotic	
Control	–	10.33	±1.25	30.33	±2.36
Myoglobin	100	10.00	±1.41	30.00	±2.45
T2N	0.1	25.67	±1.7	40.33	±3.68
	1	29.33	±2.05	46.33	±3.86
	10	62.00	±8.64	168.67	±37.28
T2N-Mb	0.1	13.00	±0.82	31.33	±1.7
	1	13.00	±1.41	34.67	±2.36
	10	19.00	±2.16	40.00	±1.63
	20	26.00	±2.16	44.67	±4.92

and its adducts are cytotoxic at concentration of 95–125 μg/ml and 200 μg/ml, respectively. These concentrations caused destruction of 50% of the cells of the tested cell lines (Table I). Unconjugated Mb was not cytotoxic even at a concentration of 2000 μg/ml. As we know, the cytotoxic effect can differ from cell line to cell line. The cytotoxic effect of free T2N can at least partially be explained by the fact that it is a reactive, conjugated lipophilic aldehyde. The much increased (ca 50-fold) cytotoxicity of the Mb adduct has no obvious explanation. In the next experiments genetic alterations in cells exposed to T2N and Mb-T2N as well as in unexposed control cells were estimated after 24h exposition, as the frequency of cells with micronuclei and of cells showing signs of chromatin condensation characteristic for apoptotic processes. The appearance of micronuclei and apoptosis are genetic events that lead to cell death (Abend *et al*., 1995). In this assay we have observed that the T2N and its Mb conjugate expressed the genotoxicity. The number of micronuclei in human K_{562} cells reached 26 ± 2.16 promille (MN/1000 cells), comparing to 62 ± 8.64 MN/1000 cells for the reference free T2N, whereas a control value was 10.33 ± 1.25 MN/1000 cells. The studied compounds expressed also the apoptotic effect in K_{562} cells as the number of apoptotic cells increased to 44.67 ± 4.92 promille for T2N-Mb, comparing to 169.67 ± 37.28 promille for free T2N, whereas a control value was 30.33 ± 1.36 promille for Mb.

These results should, however, alert food producers and consumers since some cell types can be more sensitive than the cell lines used in this test and prolonged exposure may cause toxic effects at much lower concentration. To date the knowledge about the cytotoxic properties of T2N and its adducts in different beers is very scarce. A major reason for this is the lack of simple, sensitive and specific analytical method for the determination of T2N and T2N-adducts in different food processes such as for example beer brewing. When beer shows an unpleasant taste (this happens already at ppb-levels of T2N), then most consumers discard it, while the consumer most likely will not detect the T2N that is hidden in the form of T2N-adducts. The beer brewing process consists of malting, kilning, mashing, wort production and fermentation with yeast. Each of the steps before fermentation is considered to be a T2N generating step. Tressl *et al*. (1979) and Drost *et al.* (1990) have found 200–400 μg/kg (1430–2860 nmoles/kg) of T2N in kilned malt. It is likely that the part of that T2N is transferred during mashing into the wort and finally ends up in the beer. If the mashing-in step, is carried out at lower temperature or at higher pH, the pool of T2N increases substantially (Back *et al.*, 1999). Boiling of the wort generates additional T2N (Noel *et al.*, 1999, Lermusieau *et al.*, 1999). During boiling free volatiles including T2N are partly evaporated, but the T2N-adducts are well soluble in water and they are not volatile and therefore they will stay during the mashing process.

Drost *et al.* (1990) have defined the potential of wort to form trans-2-nonenal as "nonenal potential" and found that this potential correlates with the lipoxygenase-1 (LOX-1) activity of the malt. The "nonenal potential" is supposed to reflect the total T2N. The lack of reliable methodology to quantify T2N adducts hampers also the progress of studies of the enzymatic and nonenzymatic transformation of hydroxyperoxides into T2N during different food processes such as brewing.

During fermentation any free T2N present in the wort is rapidly interacting with bisulphite. T2N-bisulphite adducts, however, represent only neglectable amount of T2N adducts formed during malting and

mashing process. Model bisulfite T2N was synthesized and characterized with ^{1}H NMR spectroscopy and LC-MS by Nyborg *et al.* (1999).

It is important to be alert to the fact that beer can carry a headed toxic substance, which might affect the health, apart of the alcohol present in this beverage. Our findings for the free T2N support the finding for the HNE, and introduce a new observation which is the cytotoxicity and genotoxicity of T2N-Mb adducts, with a relatively large molecule. These observations asks for an alarm clock to worn the consumer and the industrialists how to control and prevent the presence of such substance in food products.

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