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Effect of food components and processing parameters on DNA degradation in food

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The effect of food components on degradation of DNA by DNase I (EC 3.1.21.1) was monitored by electrotransformation of *Escherichia coli*, making it possible to determine the number of plasmid molecules capable of giving rise to transformed cells. The transformation frequency increased linearly with the plasmid number within the range of 2×10^6 to 2×10^{10}. DNA degradation was reduced by one order of magnitude in the presence of 0.05% (w.v^{-1}) maltol or 1 mM putrescine. Complete inhibition of degradation was observed with ≥0.2% (w.v^{-1}) maltol, ≥0.01% (w.v^{-1}) octyl gallate or ≥0.5 mM of spermine. To monitor degradation of plant DNA during food processing, a real-time PCR system was established. The ratio of copy numbers of a potato *gbss* DNA fragment of 325 bp and a nested 96 bp fragment was determined. The latter served as internal reference for normalization. The system made it possible to exclude process-dependent changes of DNA concentration in the food matrix. Processing of genetically modified potatoes to dried potato sticks, crisps or flakes was studied and drying steps were shown to exert the strongest effect on DNA degradation, resulting in a drop of the ratio from 0.73 to 0.16.

Keywords: food processing / food components / potato / DNA stability / real-time PCR

INTRODUCTION

The safety assessment of genetically modified (GM) plants requires a holistic approach to identify and characterize hazards that are associated with the genetic modification. As part of this approach, the likelihood of transfer of recombinant plant DNA to bacteria and its potential impact are of special interest (Codex Alimentarius Commission, 2003a, 2003b; FAO/WHO, 2000). To apply the principle of risk assessment (Codex Alimentarius Commission, 1999, 2003b; Lammerding, 1997), one has to bear in mind that horizontal gene transfer from GM plants to bacteria takes place by transformation only. Therefore, data are needed about competence development of bacteria in food, accessibility of DNA to the competent cells, frequencies of transformation, and degradation of DNA in food. It has been shown that food associated bacteria can acquire natural competence during growth and/or become naturally transformed in foods, *e.g. Bacillus subtilis* (Bräutigam et al., 1997; Kharazmi et al., 2002; Wittke et al., 2002; Zenz et al., 1998), *Escherichia coli* (Bauer et al., 1999), *Streptococcus gordonii* (Kharazmi et al., 2003b). Moreover, transfer of recombinant plant DNA to food-associated bacteria was detected, at least under optimal laboratory conditions (Kharazmi et al., 2003a). It was observed that to obtain measurable events, homologous sequences present in the DNA of the GM-plant and bacterium are required. Furthermore, such homologous sequences may facilitate co-transfer of non-homologous foreign DNA (De Vries et al., 2001; De Vries and Wackernagel 2002; Gebhard and Smalla, 1998).

In the course of food processing, DNA is subjected to degradation, reducing the number of DNA molecules that are capable to give rise to a transformed cell (Jonas et al., 2001; Rizzi et al., 2003). As a consequence, the likelihood of transformation events can decrease to levels far below the limit of experimental detection (Kharazmi et al., 2003a). The extent of DNA degradation is affected by the conditions of food processing and storage, as well as the food matrix (Rizzi et al., 2003). Straub et al. (1999)

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monitored the enzymatic degradation of plasmid DNA in summer sausages and observed protective effects of the meat matrix on DNA. Furthermore, adsorption of DNA onto particles such as clay (remnant of harvesting) can result in less susceptibility to degradation than in solution (Demaneche et al., 2001; England et al., 1998). Thus, interactions of food components with DNA may result in enhanced degradation or protection of DNA, however up to now only limited data on that process are available. Polyamines such as spermine were identified to exhibit protective effects on DNA against irradiation (Douki et al., 2000; Oh and Kim, 1998). Heat treatment and pH were identified as the main degrading parameters in food, with regard to processing condition (Bauer et al., 2003; Hupfer et al., 1998). Klein et al. (1998) monitored the degradation of DNA during production of sugar by spiking experiments, and determined the overall efficacy of DNA elimination in this process to $>10^{14}$. Gryson et al. (2002) showed that the degumming process during refining of soybean oil was crucial in removing DNA.

We investigated the effect of food components on the enzymatic degradation of DNA using a detection system based on electrotransformation of *E. coli*. This system made it possible to quantify functional DNA molecules that are capable to give rise to a transformed cell. Furthermore, we developed a monitoring system based on real-time PCR to determine independently of changes in the food matrix the extent of DNA degradation in different steps of processing. This monitoring system was used to investigate the degradation of plant DNA during processing of potato products.

RESULTS

Effect of food components on DNA degradation

To investigate the effect of food components on enzymatic degradation of DNA, a model system was established consisting of DNA of plasmid pSG100 and DNase I. Degradation was monitored by transformation of *E. coli,* making it possible to determine the number of plasmid molecules capable of giving rise to transformed cells. Therefore, the dependency of transformation frequency (TF) on the concentration of intact pSG100 molecules was investigated. The TF correlated linearly with the molecule concentration within the range of 2×10^6 to 2×10^{10} molecules.µl^{-1} (Fig. 1). Use of lower concentrations in the assay resulted in numbers of <30 CFU per plate, which are statistically not relevant. At higher concentrations, the TF approached the maximum TF given

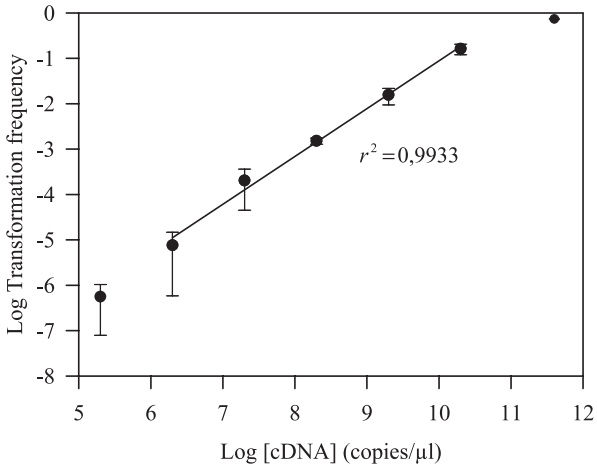

Figure 1. Dependency of the transformation frequency (TF) of *E. coli* XL1 on the concentration of molecules of plasmid pSG100. Error bars indicate standard deviations of at least 3 independent experiments.

by the maximum number of cells becoming electrocompetent. To determine the optimal incubation time with DNase I in the model, its effect on the reduction of TF over time was investigated. Within 30 s, the TF rapidly decreased by 2.5 logs, down to approximately 10^{-5}, and thereafter no further decrease was observed (Fig. 2). As the use of DNA linearized with *Pst*I resulted in comparable low TFs, it can be assumed that within 30 s, all plasmid molecules had become linearized by DNase I. However, within this time span, the determination of the TF is rather inaccurate, as it is indicated by the standard deviation. Moreover, monitoring the effect of food components on DNA degradation at 60 s also resulted in greatly varying TF values with standard errors of *ca.* 80% of the mean values (data not shown). Thus, to ensure that the monitoring of degradation is not susceptible to minor variations of DNase I activity or interactions between food component and plasmid DNA, we used a 2 min incubation time, leading to the reduction of the standard error to *ca.* 40% of the mean value.

The effect of numerous food components on the enzymatic degradation of plasmid DNA was investigated using the established model system. Out of 29 components, only maltol, octyl gallate, putrescine, and spermine affected DNA degradation, as compared to the control. No effect of the food components on the transformation event itself was observed. A concentration of only 0.05% w.v^{-1} maltol already reduced the degradation of DNA by approximately one order of magnitude, and concentrations ≥0.2% w.v^{-1} inhibited degradation completely.

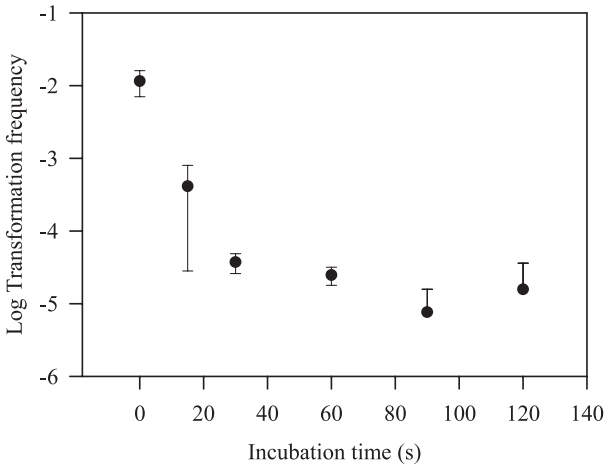

Figure 2. Degradation of plasmid DNA by DNase I monitored by determination of the transformation frequency (TF) using *E. coli* XL1 Blue. Error bars indicate standard deviations of at least 2 independent experiments.

Concentrations of 1 mM putrescine reduced the degradation by approximately one order of magnitude, and concentrations of 0.01% w.v^{-1} of octyl gallate caused complete inhibition. As a control, DNA protection by spermine was used, and concentrations of ≥0.5 mM inhibited DNA degradation completely.

Effect of pH and temperature on the degradation of plasmid DNA

During food processing, the matrix is subjected to major changes affecting the extractability of DNA and its concentration in the food. To monitor DNA degradation independently of processing and matrix effects, the following detection system was applied. Isolated DNA was subjected to real-time PCR, and the copy number of a large and small DNA fragment was determined by external calibration. As the likelihood of strand breaks increases with increasing length of DNA fragments, DNA degradation results in a reduced detectability of the large fragment compared to the small fragment (internal reference), which is also weakly degraded. Thus, the shift of ratio of the copy numbers of both fragments is correlated with the extent of DNA degradation.

To validate the applicability of this detection system, initial experiments were performed using plasmid DNA. DNA solutions were incubated at 85, 90, 95 or 100 °C at various pH values up to 20 min. Fragments of 846 bp and 516 bp were amplified and their copy number was determined. Plotting of the ratios revealed enhanced DNA degradation at increasing temperatures and decreasing pH. Examples are depicted in Figure 3, showing the combined effect of temperature and pH. No DNA degradation was observed at 85 °C / pH 7 (data not shown). These results demonstrated the applicability of the detection system. However, DNA degradation under harsh conditions (*e.g.* 100 °C / pH 5.8) can only be observed for a limited time span, since the sensitivity of the two DNA fragments to strand breaks is too high. This conclusion was reached by plotting the copy number of the 516 bp DNA fragments against the time of incubation at 100 °C (Fig. 4). At pH 6.0 the copy number decreased by approximately 6 orders of magnitude within 12 min.

Effect of food processing on the degradation of plant DNA

To investigate the degradation of plant DNA during food processing, potato sticks, flakes and chips were produced under industrial conditions using the Apriori potato. For monitoring the extent of degradation, the ratio detection system was applied. Initial studies showed that all production processes led to strong DNA degradation, since DNA fragments of 719 bp could no longer be amplified in the final products (data not shown). Thus, real-time PCR was used to amplify fragments of 325 bp and 96 bp of the *gbss* gene, since reduction of the length of DNA fragment increases their detectability under harsh degradation conditions. The PCR systems were applied to DNA isolated from the various steps of processing. With regard to processing of potato sticks, it was observed that the drying step contributes most to the degradation of plant DNA (Fig. 5). Furthermore, in the course of chip and flake production, the plant DNA was strongly degraded by the heat treatment steps (175 °C/3 min and 150 °C/1 min, respectively), and only DNA fragments of 96 bp were amplified from the DNA isolated from the final products (data not shown). Because only small DNA fragments could be amplified, the ratio detection system could not be applied in these cases.

To investigate the effect of irradiation on DNA degradation in foods, potatoes as well as intermediate and final products of potato stick production were subjected to irradiation with doses of 10, 25 or 50 kGy. Again the ratio system could no longer be applied to all steps of processing, due to the failure in amplification of the 325 bp DNA fragment. Nevertheless, for sliced potatoes, a reduction of the ratio by 4.4-fold compared to the control was observed when a dose of 10 kGy was applied. Increasing the dose only resulted in minor changes of the ratio, *e.g.* 5.7-fold for 50 kGy.

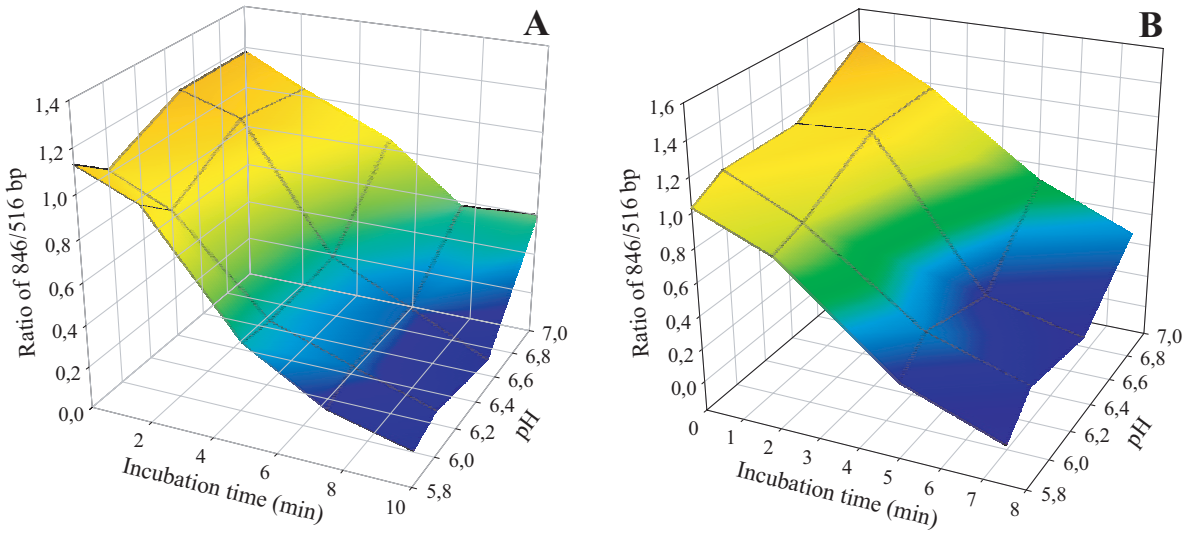

Figure 3. Effect of pH on degradation of plasmid DNA at 95 °C (A) and 100 °C (B) monitored by real-time PCR. Copy numbers of 846 and 516 bp DNA fragments were determined and the ratios were plotted against incubation time and pH.

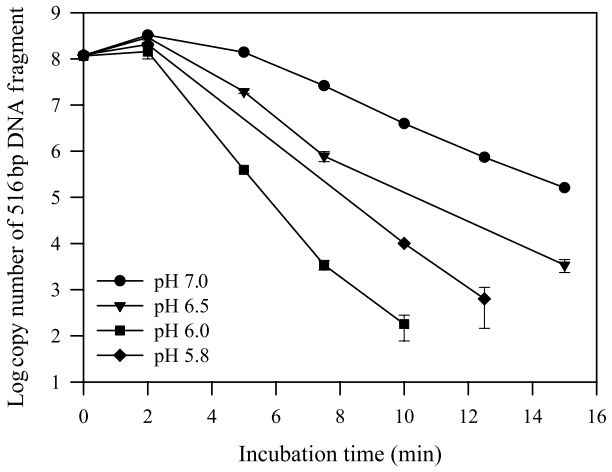

Figure 4. Degradation of the 516 bp DNA fragment of plasmid pSG100 during incubation at 100 °C and various pH values. Copy numbers (mean value ± standard deviation) were determined by triplicate real-time PCR analysis.

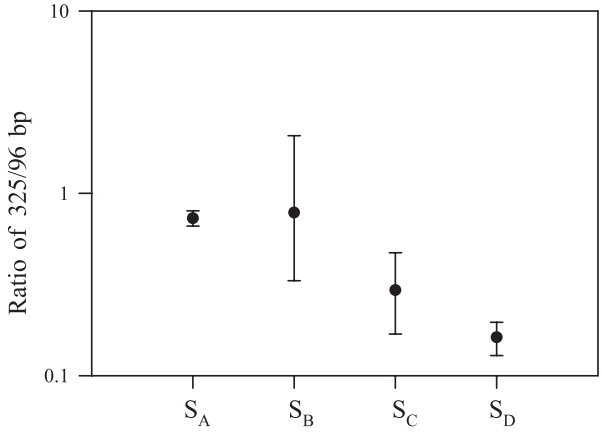

Figure 5. Degradation of plant DNA in the course of production of potato sticks monitored by real-time PCR. DNA was isolated from samples taken after slicing (S_A), blanching (S_B), drying for 2 h at 70 °C (S_C) and from the final product (S_D). Mean values are depicted, and error bars indicate the limit of variation (for calculation see Materials and Methods).

DISCUSSION

In this study, we developed novel approaches based on transformation and real-time PCR in order to measure the effect of food components and processing, respectively, on degradation of DNA in the complex environment, food. The transformation assay was designed to detect effects of a single food component on DNA degradation by nucleases, one of the major degrading factors in food (Jonas et al., 2001). Use of this assay enabled us to obtain information on DNA molecules exceeding the simple detection of their presence, since only functional molecules that are capable to give rise to a transformed cell are monitored. In contrast to the use of a marker rescue

system for monitoring the degradation of transgenic plant DNA (Kharazmi et al., 2003a); this assay has the advantage of following the kinetics of degradation over a wide range of DNA concentrations, and to perform the experiments under defined conditions, especially with regard to the time of incubation with DNase I. On the other hand, this assay does not consider potential differences in the degradation of plasmid and plant DNA molecules by DNase I.

We observed that none of the investigated food components affected the transformation efficiency of *E. coli*. In contrast to PCR-based methods, no DNA isolation or clean up was required, making the assay fast as well as reliable by reducing sources of errors. Remarkably, only 4 out of 29 representative food components exhibited a protective effect against enzymatic DNA degradation. Apparently the remaining components do not interact with the DNA and DNase I, or were applied in a concentration that is realistic with regard to foods but too low to cause effects. Maltol reduced the DNA degradation 10-fold at concentrations of ≥0.05%, equal to 500 $mg.kg^{-1}$. It arises *in situ* during food processing due to Maillard reaction, and *e.g.* approximately 40 $mg.kg^{-1}$ are naturally present in roasted coffee (Belitz and Grosch, 1992).

Octyl gallate (E 311) and other alkyl esters of gallic acid exhibit antioxidative properties and are used as food additives in amounts ranging from 0.01–0.02%. We observed protective effects of octyl gallate at concentrations of ≥0.5 mM, equal to approximately 0.014%. Recent results gave evidence for a negligible prooxidative property of octyl gallate in the presence of copper ions, which promote strand scission and formation of 8-hydroxy-2'-deoxyguanosine (Yoshino et al., 2002). Similar properties were described earlier for tannic acid, a polymer form of gallic acid (Khan and Hadi, 1998; Khan et al., 2000). With regard to the stability of DNA, these ambivalent properties make octyl gallate a highly relevant molecule. The amines putrescine and spermine exhibited protective effects at concentrations of ≥1 mM and ≥0.5 mM, equal to 0.09 $g.kg^{-1}$ and 0.101 $g.kg^{-1}$, respectively. Spermine is a key component in the cell proliferation process (Heby, 1981), and when docked into the major groove of B-DNA, stabilizes the complex by maximizing interactions between proton acceptors on DNA and proton donors on spermine (Feuerstein et al., 1986). Deng et al. (2000) demonstrated the capability of spermine and spermidine to bind and condense B-DNA without disrupting the native structure. Condensation is thought to reduce the susceptibility of DNA against shear-induced degradation (Lengsfield and Anchordoquy, 2002) which can occur in food during processing. Putrescine and spermine are widely distributed in foods and occur in concentrations above the effective ones. In fresh pork and chicken meat, concentrations of only 2.7 $mg.kg^{-1}$ were observed for putrescine (Bardócz et al., 1993). In fermented foods concentrations of putrescine of *ca.* 0.1–0.2 $g.kg^{-1}$ in sauerkraut (Halasz et al., 1994), *ca.* 0.650 $g.kg^{-1}$ in matured cheddar (Bardócz et al., 1993) and up to 3.5 $g.kg^{-1}$ in Maasdamer or Gouda (Halasz et al., 1994) were observed. Stute et al. (2002) determined the content of biogenic amines in 45 fish sauces from the Far East, and found up to 1.3 $g.kg^{-1}$ putrescine.

Until now, DNA degradation caused by food processing had been investigated using PCR amplification. However, foods undergo major changes during processing, which alter the concentration of DNA and/or the extractability of DNA by *e.g.* formation of crosslinks due to Maillard reaction, starch gelatinisation or protein denaturation. Thus, the monitoring of DNA degradation by quantification of DNA molecules is hampered by variations in yield and quality of the extracted DNA. Our ratio detection systems made it possible to overcome these limitations and to monitor degradation of plant DNA during processing of dried potato sticks (Fig. 5). The failure of application of this system to potato flake and crisp production showed that the length of amplified fragments has to be adapted to the prevailing degradation conditions. Harsh conditions as applied during potato flake and crisp production would require the amplification of DNA fragments far below 300 bp. However, a difference in the fragment length of approximately 300 bp was found to be suitable for successful application of the system (Fig. 3). Further reduction of this value would abolish differences in the degradation kinetics of DNA fragments due to similar sensitivity to the degrading factors, and changes of the ratio would become too small.

During processing of potatoes we identified heat treatment as a major parameter contributing to degradation of plant DNA. The potatoes were subjected to high temperatures (150 to 175 °C) for short times (minutes), conditions that are commonly used in drying and frying processes. Until now, DNA degradation during food production was either monitored in processes run at temperatures up to 121 °C (Bauer et al., 2003; Gryson et al., 2002; Hupfer et al., 1998, 1999; Klein et al., 1998) or detected upon investigation of final products for reasons of traceability and labelling of GM food according to the European legislation (European Commission, 1997, 1998, 2000, 2003). For example, Klein et al. (1998) showed a reduction of plasmid DNA in the course of sugar production by three orders of magnitude during heat treatment at 121 °C for 30 min. Taking into account the long exposure time, the DNA seems to be rather stable at this

temperature. On the other hand, 121°C is clearly below the extreme temperatures applied in potato crisp production. Intrinsic factors such as metal ions, pH or the formation of reactive intermediates during the heat treatment enhance the naturally occurring degradation of DNA (Lindahl, 1993). In addition, the matrix in which the free DNA is embedded plays an important role in DNA stability. For example, high persistence of bacterial DNA was observed in fermented summer sausages (Straub et al., 1999). The DNA can also be physically protected against nucleases when adsorbed to particles *e.g.* clay minerals (Demaneche et al., 2001).

In conclusion, under certain conditions, rapid degradation of DNA can occur during processing of foods. This degradation is affected by indigenous factors such as food components, pH, nucleases, etc. and exogenous factors prevailing during processing and storage, which can act in a synergistic, antagonistic or additive way. Due to the huge variability of these factors in food and food production, it is obvious that the extent of DNA degradation cannot be predicted for a defined food product or processing step.

MATERIALS AND METHODS

Bacterial strains, plasmid, potatoes and food components

Plasmid pSG100 is a derivative of pUC18 (Norrander et al., 1983) containing the kanamycin resistance gene *nptII* (Beck et al., 1982). *Escherichia coli* JM109 (Yanisch- Perron et al., 1985) and XL1 Blue (Stratagene) were used as host for propagation of pSG100 and transformation experiments, respectively, and were grown in LB medium (Sambrook and Russel, 2001) at 37 °C. Selective media contained 100 $\mu g.ml^{-1}$ ampicillin. Electrocompetent cells of *E. coli* were prepared as described by Sambrook and Russel (2001). Genetically modified *Solanum tuberosum* cv. Apriori tubers were kindly provided by Avebe (The Netherlands). The following food components (% $w.v^{-1}$, if not indicated else) were used dissolved in Tris-HCl (10 mM, pH 8.4): Acesulfame K (0.5), adonitol (1), bovine serum albumin (10, 1, 0.1 $mg.ml^{-1}$), 3-tert-butyl-4-hydroxyanisole (0.5), carboxymethylcellulose (1), k-carrageenan (0.5), collagen (0.5), creatine (0.5), curcumin (0.1), 4-hydroxybenzoic acid (0.5), myo-inositol (1), lactic acid (1), lactose (1), D-mannitol (1), maltol (2, 1.5, 1, 0.5), sodium nitrate (0.25), Patent blue V (0.1), phenylalanine (1), polyethylenimine (1, 0.5, 0.1, 0.05), putrescine (100, 10, 1 mM), saccharin (0.5), Simplesse (1, 0.5, 0.1), sorbitol (5, 1), spermine (50, 5, 0.5, 0.05 mM), tartrazine (0.1), taurine (0.5). The following food components were dissolved in ethanol/Tris (50/50, v/v): mono/di-glyceride (1), octyl gallate (5, 1, 0.5, 0.1), propyl gallate (5, 1), riboflavin (saturated).

DNA isolations

Plasmid DNA was isolated from over-night cultures using the Qiafilter Maxi Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted with Tris-HCl (10 mM, pH 8.4). The concentration of plasmid DNA solutions was determined as described by Berdal and Holst-Jensen (2001) and diluted to either 2.0×10^{10} or 2.0×10^{8} molecules.μl^{-1} and stored at 8 °C. For extraction of genomic plant DNA, potato tubers or products were milled in a mortar with liquid nitrogen. DNA was isolated from 150 mg of homogenized sample using the Nucleo-Spin Plant DNA extraction kit (Macherey-Nagel, Germany). The manufacturer's protocol was modified as follows: 800 µl of buffer C1 and 10 µl RNase A were added and incubated for 30 min at 37 °C followed by 30 min at 60 °C. After centrifugation (12 000 × *g*, 21°C, 5 min), the supernatant was transferred to a new cap and 800 µl of buffer C4 as well as 600 µl ethanol were added. The mixture was subjected to the column and centrifuged (12 000 × *g*, 21°C, 1 min). DNA was washed with 400 µl of buffer CW, 700 µl of buffer C5 and once again with 200 µl of buffer C5. DNA was eluted from the column with a total volume of 50 µl of buffer CW (preheated to 70 °C).

Monitoring DNA degradation by transformation

Dilutions of DNase I (EC 3.1.21.1, Invitrogen) were freshly prepared and adjusted to 0.5 $U.\mu l^{-1}$. In a volume of 95 µl, 10 µl of the food component solution and 2×10^{11} molecules of pSG100 were incubated in buffer (2 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl pH 8.4) together with 5 U of DNase I. The negative control was prepared without food component and DNase I. In the positive control only the food component was omitted. To exclude any effect of the food component on the transformation event itself, an additional control without DNase I but with the food component was performed. The mixture was incubated at 37 °C for 2 min and degradation was stopped by addition of Na_2EDTA to a final concentration of 2.5 mM. An aliquot of 1 µl was immediately used for electrotransformation of *E. coli* XL1 Blue, using a GenePulser Xcell (Bio-Rad) as described by Sambrook and Russel (2001). Total cell counts and counts of transformants were determined on

LB medium and SOB containing 100 µg.ml^{-1} ampicillin, respectively.

Processing of Apriori potatoes

Processing of Apriori potatoes to crisps, puree-powder (flakes) and dehydrated potato sticks was performed in the semi-technical facilities of the Federal Centre for Cereal, Potato and Lipid Research (BAGKF, Detmold, Germany) under conditions comparable to industrial production. For production of sticks, Apriori tubers were abrasive peeled, sliced to sticks of 24 × 6 × 6 mm^3 and washed with cold tap water. Sticks were blanched for 150 s and washed to remove gelatinised starch. Drying of sticks was performed in two steps: 70 °C for 2 h followed by 50 °C for 2.5 h. Samples were taken after slicing (S_A), blanching (S_B), drying step I (S_C) and from the final product (S_D). For production of flakes, tubers were abrasive peeled, sliced (10 mm thickness) and washed with cold tap water. Slices were heated to 70 °C for 20 min and conditioned at room temperature for 20 min. Thereafter, they were cooked in a steam cooker for 20 min, mashed and roll dried at 150 °C to obtain flakes. Alternatively, 5 g mono/di-glyceride (E 471) per kg potato were added to the cooked potatoes (during mashing), and flakes were produced as described above. Samples were taken after slicing (F_A), cooking (F_B) and from the final product without (F_C) and with E 471 (F_D). For production of crisps, tubers were abrasive peeled, sliced (1.2 mm thickness) and washed with cold tap water. Sliced tubers were further treated as follows: no blanching or blanched at (i) 80 °C in water for 1 min, (ii) 80 °C in 0.25% $NaHSO_3$ for 1 min, or (iii) 80 °C in 0.25% $CaCl_2$ for 1 min. Finally, crisps were fried in peanut oil at 175 °C for 3 min. Samples were taken after slicing (C_A), blanching (C_B) and after frying (C_C).

In addition, the final products and all samples taken from the steps of potato stick production as well as frozen and thawed potatoes were irradiated with 10, 25 or 50 kGy in the facilities of the Federal Research Centre for Nutrition (BfE, Karlsruhe, Germany).

Degradation of plasmid DNA by pH and heat treatment

Stock solutions of 100 mM BisTris (Sigma-Aldrich) were adjusted to pH 5.8, 6.0, 6.5 and 7.0 using HCl. In a total volume of 100 µl, 2.0 × 10^9 molecules of pSG100 were incubated in 10 mM BisTris for 0–20 min at 85, 90, 95, and 100 °C. After heat treatment the aliquots were cooled on ice and aliquots were immediately subjected to real-time PCR.

Real-time PCR systems

Real-time PCR amplifications with DNA of Apriori potato samples were carried out on an ABI 7000 Sequence detection system (Applied Biosystems). Primers GBSS-F1 (5'-TGTAGCTTGGTAGATTCCCCTTT-3'), GBSS-R1 (5'-CTAGTGAAGTTTGGCTTCTTGACA-3'), GBSS-R2 (5'-TTGCTCCAAGGACCAACCT-3'), and GBSS-R6 (5'-CTCCAAGAACATTGGGTGGT-3') targeting against the recombinant *gbss* (accession no. X83220) were constructed using Primer Express 2.0 software (Applied Biosystems) and Primer3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The PCR mix (30 µl) contained 1 × SYBR Green PCR Master mix (Applied Biosystems), 0.5 mM of each primer, 4 mM $MgCl_2$, 3 µg BSA (Roche Diagnostics), and 5 µl DNA solution. The thermal cycling program consisted of a preliminary incubation step of 2 min at 50 °C followed by an initial denaturation step at 95 °C for 10 min and 50 cycles of 95 °C for 15 s, 60 °C for 60 s and 72 °C for 60 s. A melting curve analysis was performed starting at 60°C. For amplification of PCR products of different length the following primer pairs were used: GBSS-F1/ GBSS-R1 (96 bp), GBSS-F1/GBSS-R2 (325 bp) and GBSS-F1/GBSS-R6 (719 bp). The ratio of concentrations of two DNA fragments was calculated using the mean values ($\bar{x}_{325}.\bar{x}_{96}^{-1}$). The limit of variation was determined using the mean values ($\bar{x}$) and corresponding standard deviations (s) as follows: $(\bar{x}_{325}+s_{325}).(\bar{x}_{96}-s_{96})^{-1}$ and $(\bar{x}_{325}-s_{325}).(\bar{x}_{96}+s_{96})^{-1}$.

For amplification of DNA of pSG100, the primers described previously (Bauer et al., 2003) were used and amplification was performed as described above with the following modifications: pre-incubation at 50 °C for 2 min and initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 52 °C for 60 s and 72 °C for 120 s. The following primer pairs were used: Fpri3/Rpri (516 bp) and pwlF/pwlR (846 bp).

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