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Arxula adeninivorans Recombinant Guanine Deaminase and Its Application in the Production of Food with Low Purine Content

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Key Words

Arxula adeninivorans · Hyperuricemia · Recombinant guanine deaminase · Treatment of food

Abstract

Purines of exogenous and endogenous sources are degraded to uric acid in human beings. Concentrations >6.8 mg uric acid/dl serum cause hyperuricemia and its symptoms. Pharmaceuticals and the reduction of the intake of purine-rich food are used to control uric acid levels. A novel approach to the latter proposition is the enzymatic reduction of the purine content of food by purine-degrading enzymes. Here we describe the production of recombinant guanine deaminase by the yeast *Arxula adeninivorans* LS3 and its application in food. In media supplemented with nitrogen sources hypoxanthine or adenine, *guanine deaminase* (*AGDA*) gene expression is induced and intracellular accumulation of guanine deaminase (Agdap) protein occurs. The characteristics of the guanine deaminase isolated from wild-type strain LS3 and a transgenic strain expressing the *AGDA* gene under control of the strong constitutive *TEF1* promoter were determined and compared. Both enzymes were dimeric and had temperature optima of 55°C with high substrate specificity for guanine and localisation in both the cytoplasm and vacuole of yeast. The enzyme was demonstrated to reduce levels of guanine in food. A mixture of guanine deaminase and other purine degradation enzymes will allow the reduction of purines in purine-rich foods.

Introduction

Humans absorb different amounts of RNA, DNA, nucleotides, nucleosides and free purine bases from food of animal and vegetable origin [Wolfram and Colling, 1987]. Purine degradation leads ultimately to CO_2 and ammonia, although in some organisms, intermediate molecules accumulate [Hayashi et al., 2000; Keilin, 1959; Lehninger, 2004]. In humans, purines from exogenous sources (food) and purines from cell turnover are both degraded to uric acid [Wolfram, 1992; Wolfram and Colling, 1987].

Reduced renal uric acid excretion combined with increased uric acid synthesis from endogenous and/or exogenous sources can cause an increase in serum uric acid concentration in some individuals [Kutzing and Firestein, 2008; Saag and Choi, 2006; Tausche et al., 2009]. A serum concentration >6.8 mg uric acid/dl serum is above the physiological saturation level of uric acid and is de-

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fined as hyperuricemia [Terkeltaub, 2010]. A minority of patients with hyperuricemia develop microscopic and macroscopic monosodium urate crystals, which cause swelling, inflammation and/or pain in joints and results in painful gout attacks [Sorbera et al., 2010; Terkeltaub, 2010]. Between 1 and 2% of the population in industrial countries are affected by this disease [Burns and Wortmann, 2011; Eggebeen, 2007; Sorbera et al., 2010].

Patients with acute gout attacks are treated with NSAIDs (non-steroidal anti-inflammatory drugs), colchicine or in rare cases, with corticosteroids or interleukin-1β inhibitors [Groff et al., 1990; Jordan, 2012; Tausche et al., 2009; Terkeltaub et al., 2010]. In symptomless intervals between attacks, uricostatic agents (allopurinol or febuxostat) which reduce uric acid synthesis by inhibition of xanthine oxidoreductase [Eggebeen, 2007; Hu and Tomlinson, 2008; Pascual et al., 2009; Wittköpper et al., 2011], uricosuric agents (probenecid or benzbromarone) which promote uric acid excretion [Eggebeen, 2007; Emmerson, 1996] or recombinant urate oxidase (rasburicase or pegloticase) which enzymatically degrade uric acid [Leinmüller, 2001; Schlesinger et al., 2011; Sharma and Verma, 2004; Sundy et al., 2007] are used.

The uptake of exogenous purines can be reduced by decreasing the intake of purine-rich food such as meat (especially muscle tissue, bowel and skin), fish (herring and shrimps) and some plants (soy and other legumes) [Wolfram, 1992; Wolfram and Colling, 1987]. Additionally, avoidance of alcohol and loss of weight are useful in the prevention of gout attacks [Wolfram, 1992].

Our approach to prevent hyperuricemia involves the addition of a mixture of different purine-degrading enzymes to the food, either during production or during ingestion. This study characterises one of those enzymes, guanine deaminase, and investigates its performance in a purine-rich food.

Guanine deaminase (EC 3.5.4.3, guanine aminohydrolase), is a metal-dependent enzyme that is highly conserved, and was first described in 1932 [Schmidt, 1932]. It catalyses the irreversible deamination of guanine to xanthine and ammonia [Fernández et al., 2010; Glantz and Lewis, 1978; Lehninger, 2004; Maynes et al., 2000]. It is responsible for purine degradation in bacteria [Liaw et al., 2004; Maynes et al., 2000], lower eukaryotes [Nolan, 1984; Saint-Marc and Daignan-Fornier, 2004; Shavlovskii and Kuznetsova, 1974], plants [Barankiewicz and Paszkowski, 1980; Negishi et al., 1994] and is present in the liver [Gupta and Glantz, 1985; Lewis and Glantz, 1974] and kidney [Kubo et al., 2006] of higher eukaryotes. Guanine deaminase was also found to be active in the brain of higher eukaryotes [Berger et al., 1985; Kumar and Rathi, 1980; Miyamoto et al., 1982; Rossi et al., 1978] where it plays an important role in the branching of dendrites [Fernández et al., 2009].

In this study the *guanine deaminase* (*AGDA*) gene was isolated from the non-conventional, arthroconidial, xerotolerant, anamorphic, non-pathogenic yeast, *Arxula adeninivorans* LS3 [van der Walt et al., 1990]. The yeast is able to assimilate and use a broad range of substrates, including purines, *n*-alkanes and starch as sole energy and carbon sources and nitrate as a sole nitrogen source [Böer et al., 2009c; Gienow et al., 1990; Middelhoven et al., 1984]. It can be cultured at up to 48°C and 20% NaCl [Wartmann et al., 1995; Yang et al., 2000]. Above 42°C the morphology changes from budding cells to mycelia [Wartmann et al., 1995].

The *ADGA* gene was overexpressed to produce high concentrations of recombinant guanine deaminase protein. Additionally, the application of the enzyme in a process to reduce purines in food was investigated.

Results

Identification and Characterisation of the AGDA Gene

The putative nucleotide sequence of the *AGDA* gene was determined from the complete *A. adeninivorans* sequence and annotated genome [manuscript in progress] and the *AGDA* gene was located as single copy on chromosome 4. The ORF of the *AGDA* gene comprises 1,428 bp which encodes a protein with 475 amino acids (molecular mass 53 kDa; isoelectric point 5.7). Agdap has identity with other fungal guanine deaminases, including *Ogataea parapolymorpha* DL-1 (accession: EFW97840 – 55%), *Komagataella pastoris* GS115 (XP_002490656 – 51%), *Saccharomyces cerevisiae* strains (CAY79050, EDN60126 and EEU08967 – 49–50%), *Ajellomyces dermatitidis* strains (EEQ86158 and XP_002620893 – 45%), *Schizosaccharomyces pombe* 972h- (NP_587874 – 48%) and *Paracoccidioides brasiliensis* Pb03 (EEH21407 – 44%).

Nitrogen Source-Dependent Expression of the AGDA Gene

The analysis of *AGDA* transcription regulation was done with qRT-PCR and the *Arxula* genes *AHBS4*, *ALG9*, *TEF1* and *TFIID* as stably expressed endogenous references. The wild-type strain was cultured in YMM-glucose supplemented with 43.5 mM $NH_4H_2PO_4$ as nitrogen

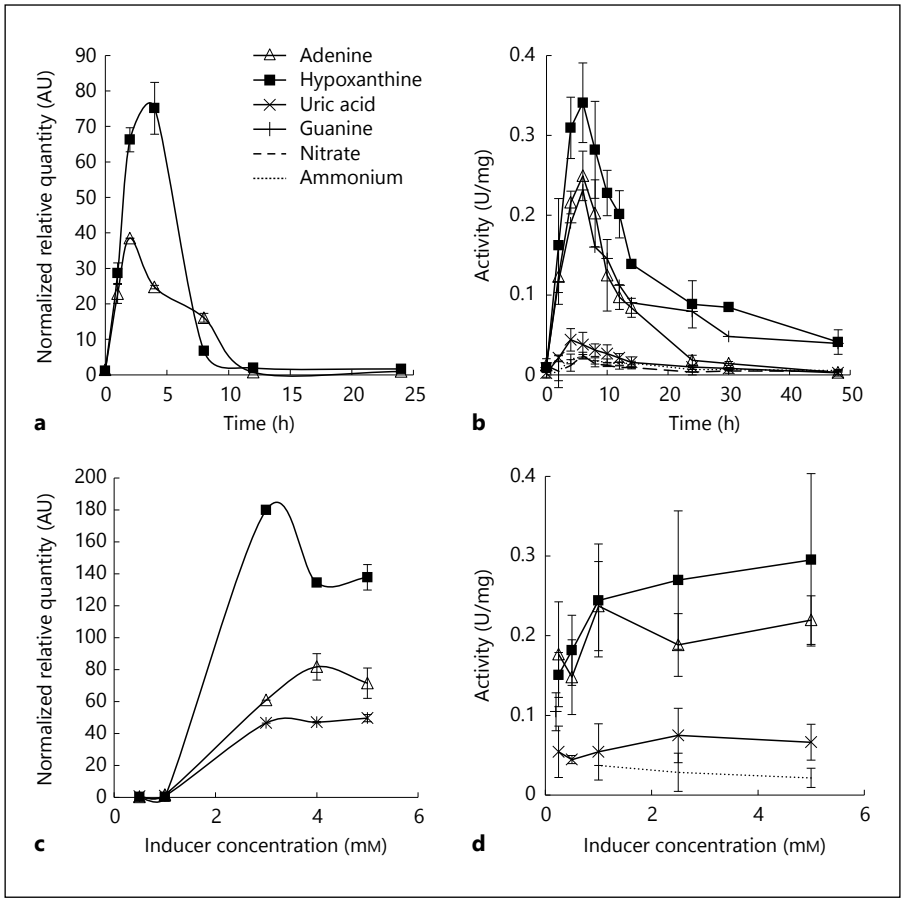

Fig. 1. Influence of the nitrogen source on *AGDA* transcript accumulation (**a**, **c**) and Agdap activity (**b**, **d**). Normalised relative quantity of *AGDA* transcript level was analysed with qRT-PCR and calculated according to Hellemans et al. [2007] with *Arxula* genes *AHBS4*, *ALG9*, *TEF1* and *TFIID* used as stably expressed endogenous references. *A. adeninivorans* LS3 was cultured 24 h at 30 °C in YMM-glucose-$NH_4H_2PO_4$ (43.5 mM) before being shifted to YMM-glucose supplemented with different nitrogen sources (**a**) 5 mM adenine, hypoxanthine or $NaNO_3$ for 24 h, (**b**) 5 mM adenine, hypoxanthine, uric acid, $NaNO_3$, $NH_4H_2PO_4$ or 0.2 mM guanine for 48 h, (**c**) 0.5, 1, 3, 4 and 5 mM adenine, hypoxanthine or $NaNO_3$ for 2 h, and (**d**) 0.25, 0.5, 1, 2.5 and 5 mM adenine, hypoxanthine, uric acid, $NH_4H_2PO_4$ or 0.2 mM guanine for 4 h.

source (N-source) for 24 h at 30°C before the washed cells were shifted to YMM-glucose containing 5 mM adenine, 5 mM hypoxanthine or 5 mM $NaNO_3$ as nitrogen source. The normalised relative quantity of *AGDA* transcript was calculated as ratio of the transcript in a sample (YMM-glucose-purine) to the transcript in the control (YMM-glucose-$NaNO_3$). Immediately after the shift of cells into media with purine as nitrogen source, the *AGDA* transcript level increased and reached maximum level at 2 h with adenine and 4 h with hypoxanthine, after which transcription fell to the basal level. The maximal transcript level was 2-fold higher in cells cultured in YMM-glucose-hypoxanthine than in cells cultured in YMM-glucose-adenine (fig. 1a).

The crude extract of *A. adeninivorans* LS3 yeast cells, cultured under the same conditions, was used for the determination of Agdap activity. After a shift from YMM-glucose-$NH_4H_2PO_4$ to YMM-glucose supplemented with 5 mM adenine, 5 mM hypoxanthine or 0.2 mM guanine as a nitrogen source, the enzymatic activity strongly increased which is in contrast to uric acid as nitrogen source where the activity increase was minimal (fig. 1b). The negative controls (YMM-glucose-$NH_4H_2PO_4$ or YMM-glucose-$NaNO_3$) showed no increase of Agdap activity. The maximal Agdap activity was achieved 6 h after the shift to adenine or hypoxanthine.

The effect of various concentrations of inducer on *AGDA* transcript level was determined by harvesting cells 2 h after a shift to YMM-glucose containing different concentrations (0.5, 1, 3, 4, 5 mM) of hypoxanthine, uric acid, adenine and $NaNO_3$ as nitrogen sources and analysing for the *AGDA* transcript. Low inducer concentrations (0.5 and 1 mM) resulted in low *AGDA* transcript levels whereas concentrations of 3 mM or higher increased the mRNA accumulation (fig. 1c) with the highest level being achieved with 3 mM hypoxanthine.

Because the low activity at 2 h, guanine deaminase activity was analysed 4 h after the shift to YMM-glucose-nitrogen sources (fig. 1d). At 4 h, Agdap activity had strongly increased with concentrations of 1 mM or greater of hypoxanthine 0.24–0.30 U/mg protein, adenine 0.15–0.22 U/mg protein and 0.2 mM guanine 0.11 U/mg

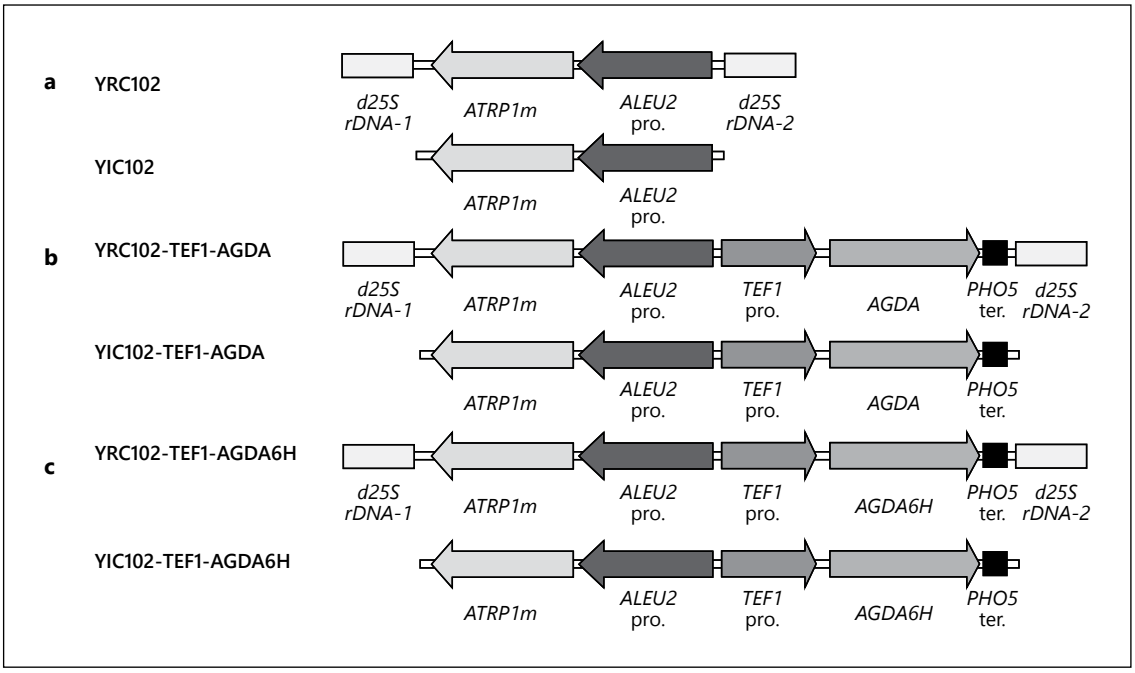

Fig. 2. Physical maps of yeast transformation/expression cassettes (YRC and YIC) used in this study. The selection module contains the selection marker *ATRP1m* fused to the *ALEU2* promoter. The selection module is fused to none (**a**) or one (**b**, **c**) expression module. The expression modules contain the following elements: *A. adeninivorans*-derived constitutive *TEF1* promoter, *AGDA*-coding sequence and *S. cerevisiae*-derived *PHO5* terminator. *AGDA*-coding sequence is fused to C-terminal HisTag (**c**) encoding sequence. YRCs are flanked by 25S rDNA sequences for targeting; YICs are without 25S rDNA targeting sequences.

protein. Uric acid as a nitrogen source again induced low Agdap activity (<0.07 U/mg protein). YMM-glucose-$NH_4H_2PO_4$ showed only weak guanine deaminase activity which was independent of $NH_4H_2PO_4$ concentration.

Production of Recombinant Guanine Deaminase, Agdap

The *AGDA* gene was overexpressed under the control of the strong constitutive *TEF1* promoter in the auxotrophic *A. adeninivorans* G1212 strain (Δ*atrp1*) using the Xplor®2 transformation/expression platform [Böer et al., 2009b]. Xplor®2 allows the construction of resistance marker-free yeast vectors with selection and expression modules as described in the Materials and Methods section. The vector for control contains only the selection module. The linear vector fragments were introduced into the *A. adeninivorans* G1212 strain as yeast rDNA integrative expression cassettes (YRC, digested with *Asc*I) and yeast integrative expression cassettes (YIC, digested with *Sbf*I) (fig. 2).

For initial screening the protein extracts from approximately 50 transformants per construct (cultured 24 h in YMM-glucose-$NaNO_3$) were analysed for recombinant Agdap or Agda6hp activity (data not shown). Increased guanine deaminase activity was observed in most transformants in comparison to the controls G1212/YIC102 and G1212/YRC102.

The transgenic strains with highest Agdap activity were analysed in time course experiments over 96 h at 30°C in YMM-glucose-$NaNO_3$. The cultivation conditions resulted in stationary growth phase being reached after approximately 24 h with all strains (fig. 3) exhibiting an increased level of intracellular Agdap or Agda6hp after 24 h (52–73 U/g dry cell weight (dcw); 320–430 U/l culture) before decreasing to basal level. The control strains, without expression modules, gave Agdap activities <5 U/g dcw, although there was no difference detectable in the biomass of transgenic strains and controls. The activity levels of YRC and YIC were also comparable. The highest activity was achieved with G1212/YRC102-TEF1-AGDA6H.

Purification and Molecular Mass Estimation

The endogenous guanine deaminase from *A. adeninivorans* LS3 was purified to homogeneity from induced cells cultured for 24 h in YMM-glucose-$NaNO_3$ before

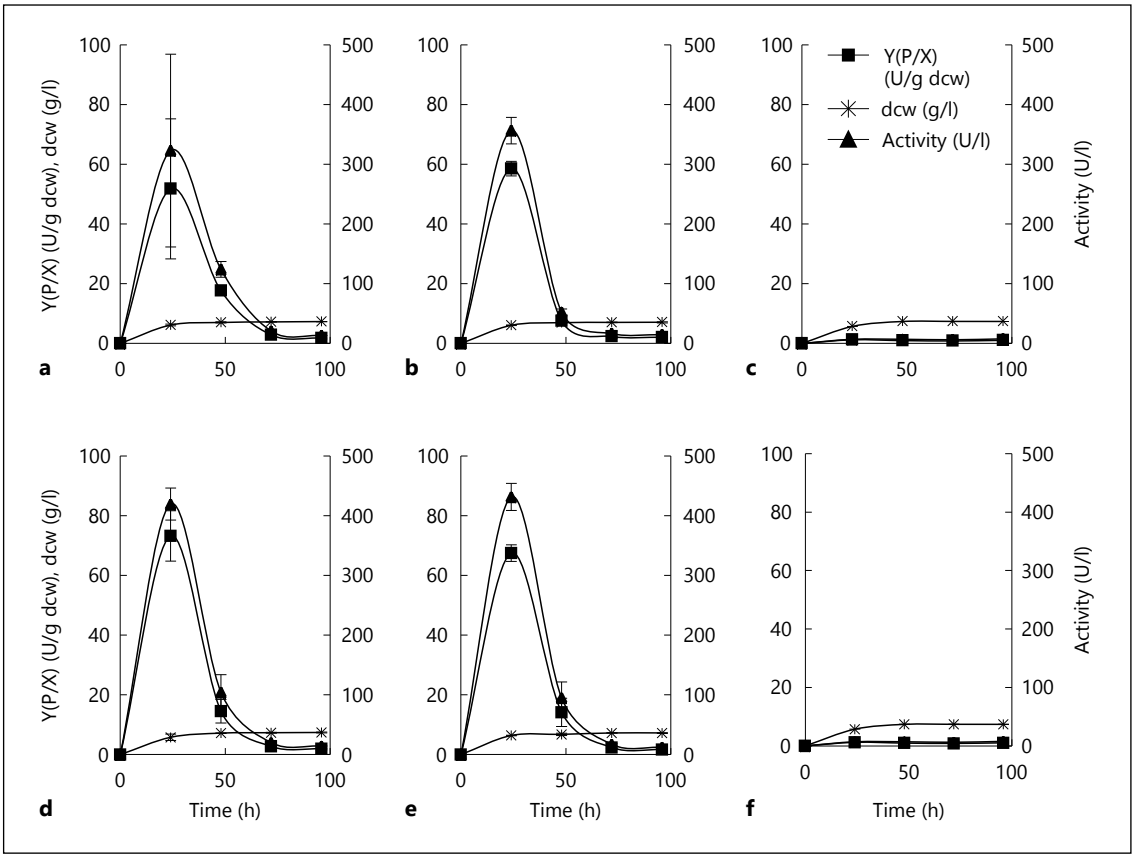

Fig. 3. Expression of guanine deaminase over time by transgenic *A. adeninivorans* strains. Transformants G1212/YIC102-TEF1-AGDA (**a**), G1212/YIC102-TEF1-AGDA6H (**b**), G1212/YIC102 (**c**), G1212/YRC102-TEF1-AGDA (**d**), G1212/YRC102-TEF1-AGDA6H (**e**) and G1212/YRC102 (**f**) were cultured in shake flasks for 96 h at 30°C in the presence of YMM-glucose-$NaNO_3$. At the indicated time, 2-ml aliquots of the culture were assayed for intracellular guanine deaminase activity (U/l culture) to determine biomass (g/l culture) and to calculate the guanine deaminase output (U/g dcw) as described in the Materials and Methods section.

Table 1. Purification steps for endogenous guanine deaminase synthesized by wild-type *A. adeninivorans* LS3

Step	Total protein, mg	Total activity, U	Specific activity, U/mg	Yield, %	Purification, fold
Crude extract	223.2	20.2	0.1	100	1
Heat precipitation	18.8	14.5	0.8	71.5	8.5
DEAE-Sepharose column	2.6	5.0	1.9	24.7	21.2
Superdex™ 200 column	0.3	1.2	4.7	6.0	51.5

being shifted to YMM-glucose-hypoxanthine (5 mM) for 6 h as described in the Materials and Methods section. The specific activity in these cells was 4.7 U/mg of protein (table 1). After the purification steps, a single protein band with an approximate molecular mass of 55 kDa was detected (fig. 4a) on denaturing SDS-PA gel and given by MALDI-TOF mass spectrometry. The protein in the band was digested with the endopeptidase trypsin into peptides, which revealed an amino acid overlay of 63% with the putative Agdap amino acid sequence from *A. adeninivorans* LS3 (fig. 4b).

The recombinant Agda6hp was purified by column chromatography on His-Bind resin as described in the Materials and Methods section, separated with denatur-

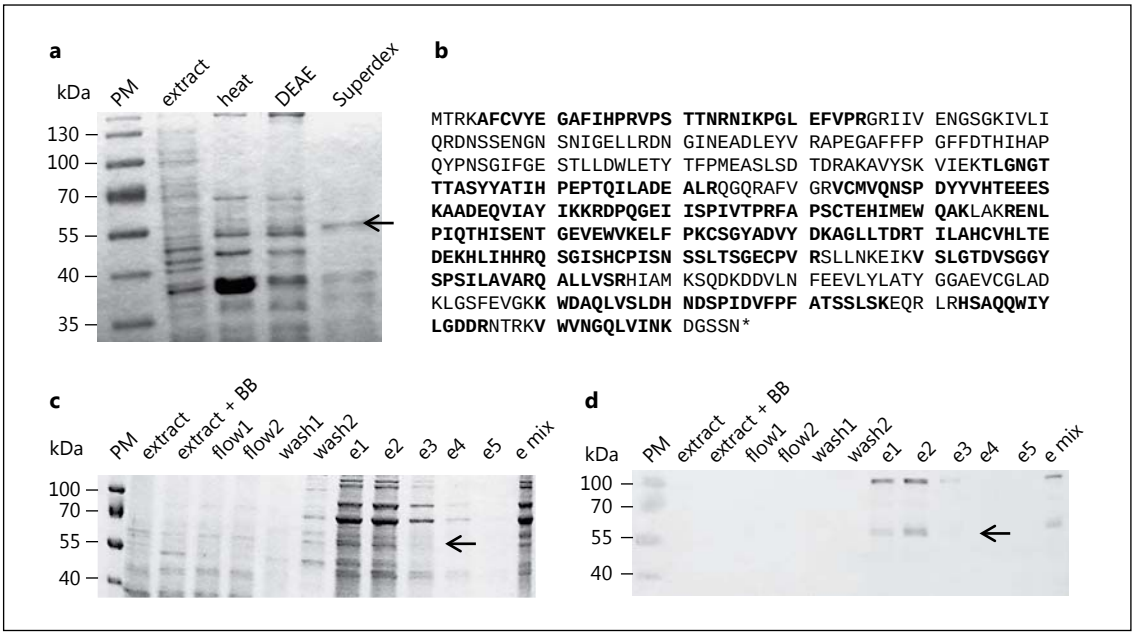

Fig. 4. Analysis of purification of guanine deaminase. **a**, **b** *A. adeninivorans* LS3 cells were cultured for 24 h at 30°C in YMM-glucose-$NaNO_3$ before being shifted to YMM-glucose-hypoxanthine (5 mM) for 6 h. After harvesting and disrupting of cells, the crude extract (extract) was purified by heat precipitation (heat), DEAE ion exchange chromatography (DEAE), Superdex™ 200 chromatography (Superdex) and electrophoresed by SDS-PAGE (12%). The marked protein band (arrow) was analysed with MALDI-TOF mass spectrometry (detected peptides are accented in bold type). **c**, **d** *A. adeninivorans* G1212/YRC102-TEF1-AGDA6H cells were cultured for 24 h in 30°C in YMM-glucose-$NaNO_3$, harvested, crude extracts prepared (extract) and purified by Ni-NTA chromatography. Following purification steps, extract with binding buffer (extract + BB), flow-through 1st (flow1), flow-through 2nd (flow2), washing with 5 mM imidazole (wash1), washing with 20 mM imidazole (wash2), eluate 1 (e1), eluate 2 (e2), eluate 3 (e3), eluate 4 (e4), eluate 5 (e5) (all eluation steps with 1 ml 1 M imidazole) and mixture of eluate 1 and 2 (e mix), were (**c**) electrophoresed on 12% SDS-PA gel and (**d**) analysed by immunoblot analysis with anti-HisTag antibodies.

ing SDS-PAGE (fig. 4c), treated with anti-HisTag-specific antibodies (fig. 4d) and detected at approximately 55 kDa.

The molecular mass of endogenous and recombinant guanine deaminase proteins were determined by gel filtration on Superdex™ 200 column with both enzymes having a molecular mass between 100 and 110 kDa (table 2). The active enzymes are probably homodimers.

Enzymatic Analysis and Subcellular Localisation of Agdap and Agda6hp

The temperature optimum was similar for endogenous and recombinant guanine deaminase (55°C) but the thermostability after incubation for 1 h was higher for endogenous Agdap (60°C) than for the recombinant Agda6hp (30°C) (table 2). The Agdap from wild-type strain has optimal pH values of 6.5 and 8.0 whereas the recombinant Agda6hp showed a single optimum of pH 6.5. Both enzymes are stable during incubation for 1 h at 37°C with pH in the range of 6.5–8.0. The Michaelis constant for both enzymes was determined by Hanes-Woolf plot and calculated to be approximately 56 μM for endogenous guanine deaminase and 26 μM for recombinant guanine deaminase.

The enzymatic activity of guanine deaminase was reduced by storage at 22 and 4°C (table 3) to be less than 50% after 28 days, while at the activity increased up to 123% with storage at –20°C. The addition of the protecting agent glycerine also had negative effects on the residual activity.

The intracellular localisation of endogenous (data not shown) and recombinant (fig. 5) guanine deaminases was analysed by sucrose density gradient centrifugation as described in the Materials and Methods section. The highest enzymatic activity was detected in fractions 17 and 18 for both enzymes; in flanking fractions the activity decreased by up to 70%. The vacuole marker enzyme, α-mannosidase, exhibited activities of more than 80% in fractions 16–18 and decreasing to 50% in flanking fractions. The enzymatic activity of cytosolic marker enzyme, glucose-6-phosphate dehydrogenase, was detectable in fractions 19–22.

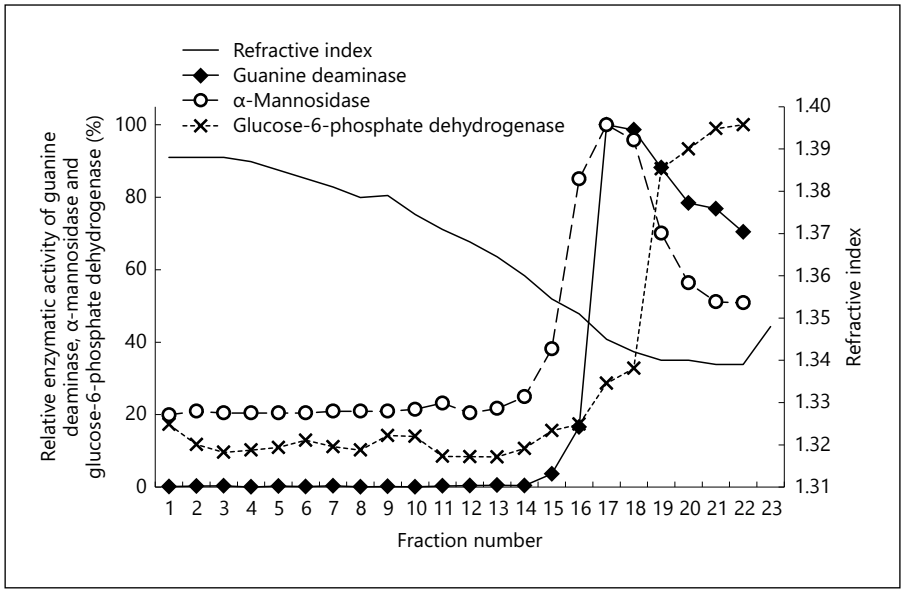

Fig. 5. Subcellular localisation of recombinant guanine deaminase Agda6hp. *A. adeninivorans* G1212/YRC102-TEF1-AGDA6H protoplasts were disrupted and loaded onto a sucrose density gradient as described in the Materials and Methods section. Each fraction was analysed for the refractive index of sucrose and enzymatic activity of guanine deaminase, the vacuole marker enzyme, α-mannosidase, and cytoplasmic marker enzyme, glucose-6-phosphate dehydrogenase.

The influence of metal ions and other reagents on endogenous Agdap and recombinant Agda6hp (table 4) was assessed and the resulting activity compared with a control without added metal ions (100% activity). The presence of Cu^{2+} or Hg^{2+} reduced the enzymatic activity of both enzymes by more than 75%. The addition of 1 mM Ni^{2+} caused a reduction in the activity for both enzymes whereas the addition of 1 mM DTT or L-cysteine reduced the activity of the recombinant guanine deaminase only. The other metals and reagents (table 4) had no effect or only weak effects at the tested concentrations.

Both guanine deaminases were analysed for substrate specificity. The highest activity of Agdap and Agda6hp was obtained with guanine (100%) while activity with 6-thioguanine and 8-azaguanine was less than 10% (data not shown). There was no activity with other purine derivatives such as adenine, adenosine and guanosine. The addition of guanosine to an enzymatic reaction with guanine as the substrate caused a decrease of enzymatic activity by approximately 50% (table 5). All the other purine derivatives that were trialled had no effect or only weak effects on the activity of endogenous Agdap and recombinant Agda6hp.

Use of Guanine Deaminase in a Mixture of Enzymes to Reduce Purines in Food

Beef stock (50 mg/ml REWE Bio Rinderbrühe; REWE, Germany), containing 35.2 mg/l guanine, 0.2 mg/l uric acid, 1.8 mg/l xanthine, 3.4 mg/l adenine and 13.4 mg/l hypoxanthine [Trautwein-Schult et al., 2013], was anal-

Table 2. Properties of the endogenous (Agdap) and recombinant guanine deaminase (Agda6hp) synthesized in *A. adeninivorans* LS3 and G1212/YRC102-TEF1-AGDA6H

Property	Agdap	Agda6hp
pH optimum	6.5 and 8.0	6.5
pH stability	6.5–8.0	6.5–8.0
Temperature optimum	55°C	55°C
Temperature stability	up to 60°C	up to 30°C
Michaelis constant, K_m	56 μM	26 μM
k_{cat}	3.9 1/s	1.5 1/s
M_r native	100–110 kDa	100–110 kDa
M_r denatured	55 kDa	55 kDa

The Michaelis constant was estimated using a Hanes-Woolf plot. pH stability was defined as the range of activity and thermal stability as highest temperature by which 80% of the initial activity was retained after an incubation time of at least 60 min. The properties were determined in at least three independent experiments with maximal aberration of 10% with standard assay for ammonia quantification.

Table 3. Stability of guanine deaminase of *A. adeninivorans* LS3 under different storage conditions

Storage condition	0 days, %	1 day, %	7 days, %	14 days, %	28 days, %
22°C	100	86	79	72	34
4°C	100	93	77	72	44
–20°C	100	100	103	117	123
–20°C with 20% glycerine	100	45	39	64	65

Table 4. Effects of various metal ions and other compounds on the stability and activity of the endogenous and recombinant guanine deaminase (Agdap and Agda6hp) synthesised in *A. adeninivorans* LS3 and G1212/YRC102-TEF1-AGDA6H respectively

Compound	Concentration, mM	Agdap activity, %	Agda6hp activity, %
Control, without compound	–	100	100
$CaCl_2$	1/0.1	103/100	100/102
$CuCl_2$	1/0.1	22/20	1/1
$CuSO_4$	1/0.1	22/23	1/2
$FeCl_3$	1/0.1	101/102	102/101
KCl	1/0.1	102/100	100/95
$MgCl_2$	1/0.1	97/103	113/109
$NiCl_2$	1/0.1	90/86	90/84
$NiSO_4$	1/0.1	90/85	90/78
ZnCl	1/0.1	103/94	100/92
$ZnSO_4$	1/0.1	98/91	97/88
EDTA	1/0.1	101/102	96/94
$HgCl_2$	1/0.1	0/1	0/1
1,10-Phenanthroline	1/0.1	97/98	104/106
DTT	1/0.1	104/103	100/80
L-Cysteine	1/0.1	108/111	98/73
PMSF	1/0.1	111/112	100/102

The enzyme was incubated for 1 h at 37°C in PP-buffer pH 6.0 containing the listed concentrations of supplements. The residual activity was measured in at least three independent experiments with a standard enzyme assay (ammonia quantification) as described in the Materials and Methods section.

Table 5. Effects of purine derivatives on endogenous (Agdap) and recombinant guanine deaminase (Agda6hp) activity

Purine	Agdap activity, %	Agda6hp activity, %
Control (without additional purine)	100	100
Adenine	102	85
Adenosine	98	91
2-Amino-6-chloropurine	93	88
8-Azaguanine	99	95
8-Azaxanthine	95	88
2,6-Diaminopurine	107	98
Guanosine	48	70
Hypoxanthine	100	87
Uric acid	89	86
Xanthine	91	87

The enzyme reaction was performed under standard conditions (ammonia quantification), except that the purine derivatives were added at the same concentration as guanine (1.3 mM) to the reaction buffer. The residual activity was measured as described in the Materials and Methods section.

ysed as described in the Materials and Methods (fig. 6a). The purine content of this product was reduced by incubation with an enzyme mixture containing 0.2 U/ml guanine deaminase, 0.2 U/ml urate oxidase, 0.2 U/ml adenine deaminase and 0.2 U/ml xanthine oxidoreductase. After incubation for 240 min at 40°C, the purine content was reduced to 3.3 mg/l guanine, 0 mg/l uric acid, 0.1 mg/l xanthine, 0 mg/l adenine and 2.5 mg/l hypoxanthine (fig. 6b).

The results of analysis of rolled fillet of ham are shown in table 6. The untreated food contained 21.13 mg/l guanine and smaller amounts of hypoxanthine (9.41 mg/l), adenine (1.38 mg/l), xanthine (1.08 mg/l) (No. 1). Uric acid was not detected. The incubation of dissolved rolled fillet of ham over 120 min or 240 min with the enzyme mixture (0.05 U/ml guanine deaminase, 0.05 U/ml urate oxidase, 0.05 U/ml adenine deaminase and 0.05 U/ml xanthine oxidoreductase) caused a reduction of the each purine in this kind of food (Nos. 3 and 4). The addition of more purine-degrading enzymes (each 0.2 U/ml) led to a faster decrease of the purine content (Nos. 6 and 7).

Discussion

Between 1 and 2% of the population in industrial countries suffers from gout, a painful result of hyperuricemia [Burns and Wortmann, 2011; Eggebeen, 2007; Sorbera et al., 2010]. In symptomless intervals between gout attacks, uricostatic or uricosuric agents and in some cases recombinant urate oxidase are given to reduce the uric acid content of serum [Eggebeen, 2007; Jordan, 2012; Leinmüller, 2001; Schlesinger et al., 2011; Tausche et al., 2009; Terkeltaub, 2010; Wittköpper et al., 2011]. Furthermore, avoidance of purine-rich food, reduction of alcohol intake and reduction of weight are helpful [Wolfram, 1992; Wolfram and Colling, 1987]. This study contributes further evidence that enzymatic reduction of the purine content of food during production or ingestion by treatment with a mixture of purine-degrading enzymes will also be a useful contributor to the reduction of hyperuricemia and its associated symptoms such as gout.

Genome data of *A. adeninivorans* LS3 and sequence data of known *AGDA* genes were used to identify the *AGDA* gene. The amino acid sequence of the 1,428-bp ORF showed higher identity (44–55%) to other fungal guanine deaminases than it did to either *Escherichia coli* deaminase or human deaminase (36% identity) [Maynes et al., 2000]. The sequence PGFFDTHIH (position 90–

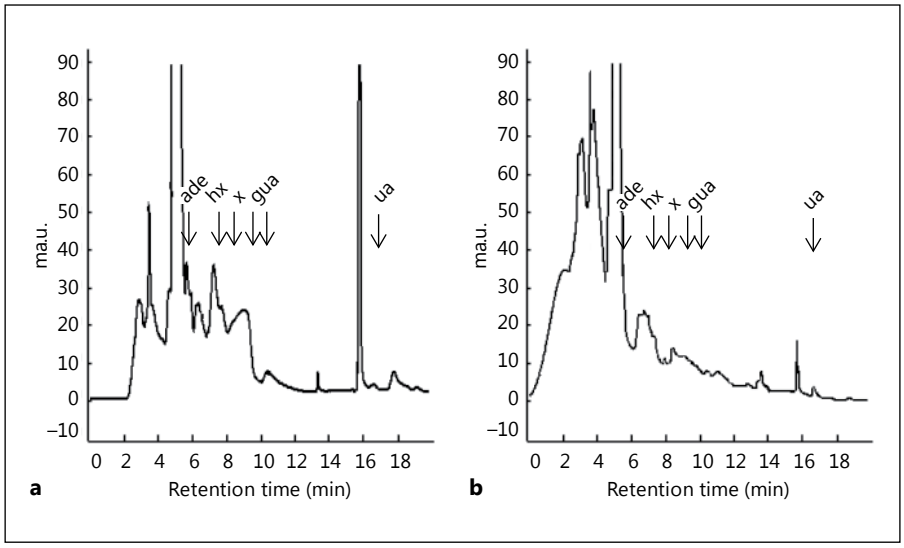

Fig. 6. HPLC analysis of beef stock incubated with purine-degrading enzymes. The profile of the beef stock solution with enzyme mix containing 0.2 U/ml guanine deaminase, 0.2 U/ml urate oxidase, 0.2 U/ml adenine deaminase and 0.2 U/ml xanthine oxidoreductase in the presence of 3 mg/ml NAD^+ (**a**) without incubation and (**b**) after 240 min at 40°C. Purine standards were used for determination of the retention time of adenine (ade), hypoxanthine (hx), xanthine (x), guanine (gua, two peaks) and uric acid (ua).

Table 6. HPLC analysis of macerated rolled fillet of ham incubated with purine-degrading enzymes. The samples were treated with different amounts of enzyme mixture and for different incubation times

Sample No.	Enzyme mix, U/ml	Incubation time, min	Purine amount, mg/l				
			adenine	hypoxanthine	xanthine	guanine	uric acid
1	0	0	1.38	9.41	1.08	21.13	0
2	0.05	0	0.77	7.35	2.90	22.07	0
3	0.05	120	0.30	0	0	0.44	0
4	0.05	240	0	0	0	0	0
5	0.2	0	0.34	6.57	3.46	17.47	0
6	0.2	120	0	0	0.32	0	0
7	0.2	240	0	0	0	0	0

98) was homologous to the conserved sequence PGX[V/I]DXH[T/V/I]H (X: each amino acid) in eukaryotic and archaeal guanine deaminases described by Fernández et al. [2009]. This sequence is common in the amidohydrolases family [Kim and Kim, 1998] and has been described in guanine deaminases of *E. coli*, *S. cerevisiae* and humans [Maynes et al., 2000; Saint-Marc and Daignan-Fornier, 2004; Snyder et al., 2000]. The amino acid sequence of *A. adeninivorans* LS3 did not, however, contain signal sequences for intracellular localisation or for secretion.

In *A. adeninivorans* LS3, the *AGDA* gene is up-regulated by adenine and hypoxanthine (maximum at 2 and 4 h respectively) although the increase of enzymatic activity was delayed in media with guanine, adenine and hypoxanthine as nitrogen sources to be at a maximum at 6 h. This effect can be explained by the higher stability of Agdap compared to the *AGDA* transcript, which is necessary to protect the cell from intracellular accumulation of purines. This is consistent with Roush et al. [1959] who observed an increase in enzymatic guanine deaminase activity of *Candida utilis* in the presence of purines. In another investigation, the promoter of *guanine deaminase* from *B. subtilis* was fused to a reporter gene [Nygaard et al., 2000]. Under nitrogen-limiting conditions the activity of the reporter gene was 10-fold higher than it is in nitrogen-rich conditions [Nygaard et al., 2000]. Additionally, an increase in the presence of adenine, hypoxanthine or guanosine as nitrogen sources was described by Nygaard et al. [2000].

The induction of mRNA transcription by purines and the nitrogen source-dependent guanine deaminase activity did not allow accumulation of the protein in wild-type *A. adeninivorans* LS3. This problem was mitigated in the

recombinant strains by using the strong constitutive *TEF1* promoter with the Xplor®2 platform as previously described for other biotechnologically interesting enzymes such as Alip1p [Böer et al., 2005], Atan1p [Böer et al., 2009a] or RR-ADH [Giersberg et al., 2012].

In this study, transgenic strains were assessed for accumulation of recombinant Agdap or Agda6hp (HisTag). The highest activity was achieved by G1212/YRC102-TEF1-AGDA6H at the end of stationary growth phase (24 h). It is possible that production of the recombinant enzyme for biotechnological applications may be increased by cultivation in fed-batch fermentation mode as observed for tannase production by *A. adeninivorans* [Böer et al., 2011].

The differences between YIC and YRC transgenic strains were small as previously described for recombinant alcohol dehydrogenase from *Rhodococcus ruber* produced in different yeast species [Giersberg et al., 2012]. In contrast, the maximal expression level of recombinant phytase K and human interferon α was achieved in YIC strains [Böer et al., 2009b]. The number of integrations was investigated with Southern blot analyses (data not shown) which showed that one transgenic strain had two integrations of the expression cassette (G1212/YRC102-TEF1-AGDA) whereas all the other transgenic strains contained a single integration. The number of integrations did not have an effect on maximal enzymatic activity of recombinant guanine deaminase, although Steinborn et al. [2007a] observed a higher expression level with increased number of integrations of α-amylase in *A. adeninivorans*. The stability of both recombinant guanine deaminases (Agdap and Agda6hp) was the same, which is in contrast to recombinant urate oxidase with a C-terminal HisTag, which increased stability of enzymatic activity when compared with urate oxidase without the C-terminal HisTag or with N-terminal HisTag [Trautwein-Schult et al., 2013].

Guanine deaminase comprises two subunits each with a molecular mass of 55 kDa. The enzyme produced by other organisms may be monomeric or dimeric [Glantz and Lewis, 1978; Maynes et al., 2000; Negishi et al., 1994; Nolan, 1984]. Guanine deaminases have been reported to have molecular masses per subunit of between 50 and 60 kDa [Bergstrom and Bieber, 1979; Gupta and Glantz, 1985; Miyamoto et al., 1982; Rossi et al., 1978], which is consistent with our findings.

The endogenous Agdap from *A. adeninivorans* LS3 has two pH optima, which is also the case for the guanine deaminase of *Camellia sinensis* [Negishi et al., 1994]. This is in contrast to guanine deaminases of other organisms, which have a single optimum between pH 6.8 and 9.4 [Bergstrom and Bieber, 1979; Fogle and Bieber, 1975; Miyamoto et al., 1982; Nolan, 1984]. The temperature optimum of the wild-type *Arxula* enzyme is relatively high at 55°C and the stability of enzymatic activity over an incubation of 1 h at 60°C is the highest ever recorded for a guanine deaminase [Glantz and Lewis, 1978; Gupta and Glantz, 1985; Nolan, 1984]. However, in contrast, the recombinant *Arxula* Agda6hp did not exhibit that level of thermostability. The reason for this and other changes in properties could be the C-terminal HisTag, which can influence protein structure and charge [Votchitseva et al., 2006]. The K_m values are similar to the values described for other guanine deaminases (4–42 μM) [Bergstrom and Bieber, 1979; Fogle and Bieber, 1975; Maynes et al., 2000; Miyamoto et al., 1982; Negishi et al., 1994; Nolan, 1984; Rossi et al., 1978].

The determination of subcellular localisation of guanine deaminase using sucrose density gradient centrifugation suggested a dual localisation in the vacuole and in the cytoplasm. In contrast, histochemical and immunohistochemical analyses of human kidney and liver assigned cytoplasmic localisation [Ito et al., 1989; Kubo et al., 2006], which was also shown by differential centrifugation in rat and mouse brain cells [Kumar et al., 1972].

The addition of ions such as calcium, magnesium or iron, which are constituents of many foods, showed no effect on the enzymatic activity of the guanine deaminase. However, toxic ions such as copper and mercury decreased the guanine deaminase activity of both the endogenous and recombinant proteins. Guanine deaminase from other organisms also showed the same inhibition with the addition of Cu^{2+} [Negishi et al., 1994] and organic mercury compounds such as *p*-chloromercuribenzoic acid and *p*-hydroxymercuribenzoic acid [Bergstrom and Bieber, 1979; Glantz and Lewis, 1978; Gupta and Glantz, 1985; Negishi et al., 1994]. These results suggest that sulfhydryl groups may be located near the active site of the enzyme. EDTA also inactivated guanine deaminase from the rat brain and *C. sinensis* [Miyamoto et al., 1982; Negishi et al., 1994]. It was shown that the loss of zinc atoms in *E. coli* leads to inactivation of the enzyme [Maynes et al., 2000]. While the guanine deaminase from *A. adeninivorans* contains the conserved zinc-binding domain (data not shown), the existence of zinc or another metal in the catalytic site of the enzyme was not proven.

The substrate spectra of guanine deaminase from *A. adeninivorans* is the same as those previously described

for guanine deaminases of human and rat liver and other organisms [Bergstrom and Bieber, 1979; Glantz and Lewis, 1978; Gupta and Glantz, 1985; Miyamoto et al., 1982; Rossi et al., 1978]. However, the inhibitory effect of guanosine was less than that observed by Kim et al. [2009]. Food contains different amounts of RNA, DNA, nucleotides and nucleosides [Wolfram and Colling, 1987] and the reduction of the negative effect of guanosine on the recombinant enzyme should be advantageous in its application for food treatment.

We have also demonstrated that the inclusion of Agda6hp in an enzyme mixture containing guanine deaminase, urate oxidase [Trautwein-Schult et al., 2013], xanthine oxidoreductase [Jankowska et al., 2013b] and adenine deaminase [Jankowska et al., 2013a] reduces the content of purines in different kinds of food. The product of a single reaction serves as substrate for another enzyme and the final product is allantoin, which is 5–10 times more soluble than uric acid. To complete our uric acid reduction system, purine nucleoside phosphatase [in progress] and non-specific phosphatases such as Apho1p [Kaur et al., 2007], an *Arxula* phosphatase of broad substrate specificity, will need to be added to the enzyme mixture to ensure the reduction purines and its derivatives in food. This approach will lead to a new application to prevent hyperuricemia.

Materials and Methods

Strains, Media and Cultivation

E. coli XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F′ proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]), obtained from Stratagene (La Jolla, Calif., USA), served as the host strain for bacterial transformation and plasmid isolation. It was grown on LB medium supplemented with ampicillin (100 μg/ml; AppliChem GmbH, Darmstadt, Germany) or kanamycin (50 μg/ml; Carl Roth GmbH, Karlsruhe, Germany) for selection.

The wild-type yeast strain, *A. adeninivorans* LS3, isolated in Siberia (Russia) from wood hydrolysates [Gienow et al., 1990] and deposited as *A. adeninivorans* SBUG 724 in the strain collection of the Department of Biology of the University of Greifswald, and the auxotrophic mutant *A. adeninivorans* G1212 [*aleu2 atrp1*::*ALEU2*] [Steinborn et al., 2007b], were used in this study.

Yeast strains were cultured at 30°C under non-selective conditions in yeast extract-peptone medium (YEPD) or under selective conditions in a yeast minimal medium supplemented with 1% (w/v) glucose (YMM-glucose) as carbon source [Tanaka et al., 1967]. The media was supplemented with $NH_4H_2PO_4$, $NaNO_3$ and a variety of purines as nitrogen sources. Agar plates were prepared by the addition of 1.6% (w/v) agar to the liquid media.

Dry cell weight (dcw) was determined by recovering cells from 1-ml samples of culture, washing twice in distilled water and drying until no further change in weight occurred.

Isolation of the AGDA Gene and Construction of Agdap Expression Plasmids in E. coli

The *AGDA* gene was identified by screening the entire *A. adeninivorans* LS3 genome for sequences that correspond to known *AGDA* gene sequences. The *A. adeninivorans* LS3 *AGDA* nucleotide sequence has been submitted to the GenBank/EMBL data libraries (HG315954).

To express a recombinant Agda protein (Agdap) in *A. adeninivorans* G1212, the *AGDA* ORF was amplified by PCR using chromosomal DNA of *A. adeninivorans* LS3 as template and primers (table 7) with the gene sequence flanked by *Eco*RI (AGDA5) and *Not*I (AGDA3) restriction sites with the primer with coding sequence for 6 histidines at the 3′-end of the ORF (AGDA3-His) used to create the HisTag proteins. The amplified *Eco*RI-*AGDA*-*Not*I and *Eco*RI-*AGDA6H*-*Not*I gene fragments, which correspond to the complete ORF, were inserted into the plasmid pBS-TEF1-PHO5-SS [Böer et al., 2005; Steinborn et al., 2006] between the *A. adeninivorans*-derived constitutive *TEF1* promoter and the *S. cerevisiae*-derived *PHO5* terminator [Arima et al., 1983; Rösel and Kunze, 1995].

The promoter-gene-terminator sequences, flanked by *Spe*I and *Sac*II restriction sites, were inserted into the plasmid Xplor2.2 [Böer et al., 2009b] to generate the plasmids, Xplor2.2-TEF1-AGDA and Xplor2.2-TEF1-AGDA6H. In the Xplor2.2 plasmid, the selection marker module (*ALEU2* promoter-*ATRP1m*) and *Eco*47III, *Spe*I, *Sac*II, *Sal*I, *Apa*I multicloning restriction sites for insertion are flanked by 25S rDNA target sequences.

The plasmids, constructed in *E. coli,* were digested with *Asc*I and *Sbf*I to remove *E. coli* sequences and the resulting linear cassettes (fig. 2) were then introduced into *A. adeninivorans* G1212.

Transformation Procedures, Isolation and Characterisation of Nucleic Acids

Transformation of *E. coli* XL1-Blue competent cells from Stratagene followed the procedure described by Inoue et al. [1990]. *A. adeninivorans* G1212 cells were transformed according to Rösel and Kunze [1998]. After passage on selective and non-selective media, stable yeast transformants were obtained [Klabunde et al., 2003]. Isolation of plasmid DNA was performed using buffers from Qiagen (Hilden, Germany). Restriction fragments were produced by the method described in the instruction manual for '*NucleoSpin® Extract II kit*' (Macherey-Nagel, Düren, Germany).

The isolation of total RNA was done with yeast cells suspended in lysis buffer (8 M guanidine hydrochloride, 20 mM MES, 20 mM EDTA, 1.25% (v/v) β-mercaptoethanol, pH 7.0) [Logemann et al., 1987]. The DNA was completely digested with DNase I (Fermentas GmbH, St. Leon-Rot, Germany) using the method described in thr manufacturer's instructions. cDNA was synthesised following the methods provided in the instruction manual '*Revert Aid™H Minus – First Strand cDNA Synthesis*' (Fermentas GmbH).

In order to quantify the *AGDA* transcript accumulation by qRT-PCR, appropriate fragments were amplified by PCR using '*POWER SYBR® Green PCR*' master mix (Applied Biosystems, Inc., Forest City, Calif., USA) in '*ABI PRISM 7700 Sequence Detection*' system (Applied Biosystems, Inc.) using the primers listed in table 7. The relative expression level of *AGDA* mRNA was calculated as described in Hellemans et al. [2007] with *Arxula* genes *AHBS4*, *ALG9*, *TEF1* and *TFIID* as stably expressed endogenous references.

Table 7. PCR and qRT-PCR primers used in the construction of the plasmids pBS-TEF1-AGDA-PHO5-SS and pBS-TEF1-AGDA6H-PHO5-SS and in the calculation of *AGDA* transcript accumulation in *A. adeninivorans* LS3

Name	Nucleotide sequence	Feature
AGDA5	5′-ATTAGAATTC**ATGACTCGAAAGGCGTTTTGC**-3′	*Eco*RI restriction site
AGDA3	5′-TAATGCGGCCGC**TTAGTTTGAGGATCCATCTTTG**-3′	*Not*I restriction site
AGDA3-His	5′-TAATGCGGCCGC**TTA***ATGGTGATGGTGATGATG***GTT TGAGGATCCATCTTTG**-3′	*Not*I restriction site and coding sequence for 6 histidines
AGDA-fw-rtPCR	5′-**GATGACCGTAACACTCGCA**-3′	Amplification in qRT-PCR
AGDA-rv-rtPCR	5′-**CGTACAGCAGAGGTCGAGA**-3′	Amplification in qRT-PCR
AHSB4-fw-rtPCR	5′-**CTCATTTATGAGGAGACCCG**-3′	Amplification in qRT-PCR
AHSB4-rv-rtPCR	5′-**ATAAAGAGTTCGTCCCTGTC**-3′	Amplification in qRT-PCR
ALG9-fw-rtPCR	5′-**CATGGGCCAAGGTATACTG**-3′	Amplification in qRT-PCR
ALG9-rv-rtPCR	5′-**AGAATGCGACCGATACCA**-3′	Amplification in qRT-PCR
TEF1-fw-rtPCR	5′-**CTCTTGGACGATTCGCC**-3′	Amplification in qRT-PCR
TEF1-rv-rtPCR	5′-**CCGTTACCGACAATCTATTT**-3′	Amplification in qRT-PCR
TFIID-fw-rtPCR	5′-**AGTTTGTGTCTGATATTGCCTC**-3′	Amplification in qRT-PCR
TFIID-rv-rtPCR	5′-**GAAGAGCTTGCTACCGAACT**-3′	Amplification in qRT-PCR

*Eco*RI (GAATTC) and *Not*I (GCGGCCGC) restriction sites are underlined; the sequences coding the 6 histidines are given in *italic type*; the sequence corresponding to the ORFs is given in bold type.

Enzyme Assays and Protein Analyses

Cells were harvested, washed, suspended in 500 µl 0.1 M potassium phosphate buffer pH 6.5 (PP-buffer) with 500 µl of silica beads (0.5 mm; BioSpec Products, Bartlesville, Okla.,USA) and shaken for 3 min at 30 Hz in Mixer Mill (MM 400; Retsch GmbH, Haan, Germany). The supernatant was separated from cell material and silica beads by centrifuging at 11,000 *g*, 10 min, 4°C and used for analysis.

Guanine deaminase activity was assayed by quantification of (A) guanine and (B) ammonia. For (A), the enzymatic activity was determined spectrophotometrically by monitoring the decrease in absorbance at 250 nm using the coefficient 9.0×10^3 l/(mol cm) [Kalckar, 1947]. Standard assay mixtures containing the sample, 0.1 M PP-buffer pH 8.0 and 0.2 mM guanine were incubated at 37°C. One unit of guanine deaminase activity was defined as the ability to catalyse the transformation of 1 µmol guanine to xanthine per minute at 37°C. The ammonia quantification (B) was performed by a modified of a procedure described by Caraway [1966]. The sample was heated to 55°C (10 min) in 0.4 ml 0.1 M PP-buffer pH 6.0 and the reaction initiated by adding 100 µl guanine (1.3 mM). The reaction was incubated for 20 min at 55°C and stopped with the addition of 0.1 ml 1.8% sulphuric acid. In the control, guanine was added after this step. Equal volumes of colour reagent (4.4% (v/v) phenol, 0.025% (w/v) sodium nitroprusside) and alkaline solution (2.5% (w/v) NaOH, 0.21% (v/v) sodium hypochlorite) was added to the clear supernatant followed by incubation for 15 min at 37°C. The assay was measured at 630 nm. One unit of guanine deaminase was defined as the amount of enzyme required to deaminate 1 µmol guanine per minute at 37°C.

Glucose-6-phosphate dehydrogenase activity was measured according to Gibon et al. [2004] and the α-mannosidase assay was performed as described by Opheim [1978].

Purified endogenous guanine deaminase was from *A. adeninivorans* LS3 cells that were cultured for 24 h in YMM-glucose supplemented with 43.5 mM $NaNO_3$ at 30°C, washed and shifted to YMM-glucose supplemented with 5 mM hypoxanthine as the nitrogen source for 6 h. Transformants were cultivated 24 h in YMM-glucose-$NaNO_3$. Cells were harvested, suspended in 0.1 M PP-buffer pH 6.5 and disrupted using Mill MM 400 mixer (Retsch GmbH) with silica beads.

The crude extract of guanine deaminase was incubated for 30 min at 60°C and precipitated proteins were separated by centrifugation (10,000 *g*, 15 min, 4°C). The clear supernatant was purified to homogeneity by DEAE-Sepharose column chromatography and gel filtration on Superdex™ 200 column [Böer et al., 2009a]. The calibration standards were dextran blue (M_r 2,000 kDa), ferritin (M_r 443 kDa), catalase (M_r 232 kDa), alcohol dehydrogenase (M_r 150 kDa), bovine serum albumin (BSA) (M_r 67 kDa) and ovalbumin (M_r 42.8 kDa).

Recombinant guanine deaminase with HisTag (Agda6hp) was purified by column chromatography on His-Bind resin (Novagen, Inc., Madison, Wisc., USA) according to the manufacturer's instructions.

Endogenous and recombinant guanine deaminase were electrophoretically separated and analysed by SDS-PAGE under denatur-

ing conditions [Laemmli, 1970]. Proteins were blotted to polyvinylidene difluoride membranes (Millipore Corp., Billerica, Mass., USA) according to Towbin et al. [1979] and treated with anti-HisTag-specific antibodies according the manufacturer's instructions.

The dye-binding method of Bradford [1976] was used for protein quantification using BSA as standard.

Subcellular Fractionation

A. adeninivorans LS3 and transgenic *A. adeninivorans* G1212 strains were cultured in YMM-glucose supplemented with 2.5 mM adenine or 43.5 mM $NaNO_3$ as nitrogen sources respectively. The cells were inoculated at an $OD_{600\ nm}$ of about 0.1 and grown to $OD_{600\ nm}$ 1.2 and harvested by centrifugation (5,000 *g*, 5 min, 22°C). They were then washed in 0.9% NaCl and incubated in 10 ml VBM solution (10 mM Tris, pH 9.0, 5 mM EDTA, 1% β-mercaptoethanol) for 10 min at 22°C. The cells were then washed twice with 0.9% NaCl before incubation in 1 M sorbitol supplemented with 0.3% (w/v) lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich Corp., St. Louis, Mo., USA) at 22°C until at least 75% of cells became protoplasts [Maráz and Šubík, 1981].

The protoplast preparation was centrifuged (2,500 *g*, 5 min, 4°C), washed twice in 1 M sorbitol and then suspended in 0.4 ml 1.8 M sorbitol with 3.2 ml 10 mM Tris pH 7.5 [Bryant and Boyd, 1995]. Cell debris was removed by centrifugation at 1,000 *g* (5 min, 4°C), and the supernatant was loaded onto a 17.5–40% sucrose density gradient and separated by ultracentrifugation in a Beckman L7-65 (Beckman Instruments, Fullerton, Calif., USA) in SW40 Ti-Rotor for 100,000 *g*, 150 min, 4°C. 0.5-ml fractions were collected from along the length of the tube.

Degradation of Purines in Food

One gram of beef stock or 200 mg rolled fillet of ham was crushed, dissolved in 1.5 or 0.3 ml 0.5 M NaOH and 18.5 or 1.2 ml 0.1 M PP-buffer pH 3.0 and treated with an enzyme mixture. The enzyme mixture contained guanine deaminase, urate oxidase [Trautwein-Schult et al., 2013], adenine deaminase [Jankowska et al., 2013a] and xanthine oxidoreductase [Jankowska et al., 2013b]. The sample was incubated at 40°C in the presence of 3 mg/ml NAD^+. The reaction was stopped by heating at 100°C for 5 min. After centrifugation, 20-µl samples of supernatant were loaded onto an HPLC system (Hitachi LaChrom Elite® L-2130; Merck, Darmstadt, Germany) equipped with a TSKgel Amide-80 analytical column (250 × 4.6 mm; Tosoh, Tokyo, Japan) and fractionated by isocratic elution in acetonitrile and 0.1% trifluoroacetic acid (85:15 v/v) at a flow rate of 1.2 ml/min over a period of 20 min at 22°C. Detection was at 260 nm and the purines were identified by comparison to the retention times of pure uric acid, guanine, xanthine, hypoxanthine and adenine.

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