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Discovery of a bacterial gene cluster for deglycosylation of the toxic potato steroidal glycoalkaloids α-chaconine and α-solanine

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**ABSTRACT**

Potato juice is a by-product of starch processing currently used as feed. However, potato proteins are an untapped source of high-protein food for human nutrition if harmful constituents notably glycoalkaloids (GAs) are detoxified. The two principle GAs found in potato are α-chaconine and α-solanine, both consisting of a solanidine aglycone with a carbohydrate side-chain. The first step in the detoxification of these compounds is removal of the trisaccharide. Whole-genome sequencing of a bacterial isolate, *Arthrobacter* sp. S41, that degrade completely α-chaconine and α-solanine revealed the presence of a gene cluster possibly involved in the deglycosylation of GAs. Functional characterization confirmed the enzymatic activity of the gene cluster involved in the complete deglycosylation of both α-chaconine and α-solanine. The novel enzymes described here may find value in the bioconversion of feed proteins to food proteins suitable for human nutrition.

**Keywords:** *Arthrobacter*; glycoalkaloids degradation; α-chaconine; α-solanine; solanidine; β-galactosidase; β-glucosidase; α-rhamnosidase; potato proteins; human nutrition
1. Introduction

As the human population continues to grow, projected to reach 9.5 billion by 2050, there is increasing pressure on our planet’s resources to provide food to feed us all\(^1\). However, the increasing demand for animal-based protein negatively impacts the environment requiring more land and water usage. Consequently, there is not only a need for more food but also for alternative protein sources that can be produced using more sustainable production practices\(^1\). The current global increase in the demand for protein is in part fueled by several socio-economic changes (e.g. increased urbanization, increased incomes) including recognition of the important role protein plays in a healthy diet\(^2,3\). Furthermore, an increased consumer awareness of the negative impact on the environment from animal-derived protein production is also driving the request for alternative protein sources\(^1\).

Potato protein extracted from the production of potato starch is a sustainable potential alternative to animal protein. Potato fruit juice (PFJ) has a high nutritional value, including higher essential amino acids (e.g. lysine, phenylalanine, tyrosine) compared to other proteins such as soy protein, and a high digestibility\(^4,5\). The market demand for alternative plant-based proteins is growing rapidly with protein ingredients today mainly based on soy, wheat and milk. Plant-based proteins have diverse application in food products including cheese, dairy products, egg products, baking goods and mixes, gelatins, meat products among others. However, many vegetable proteins, including potato, contain anti-nutritional components and thus must be refined prior to human consumption. Among the antinutritional factors present in potato, are glycoalkaloids (GAs).

GAs are secondary metabolites produced by potatoes that are toxic to pests and also to humans. GAs are produced in all parts of the potato plant but are highly concentrated in the flowers, leaves, tubers.
and fruits. If consumed in high amounts, ingestion of GAs can lead to nausea, fever, diarrhea, headache and hallucinations. Potato GAs consist of the solanidine aglycone with a carbohydrate side-chain, thought to be important for mediating interactions with cell membranes. In potato, the two main GAs are α-chaconine and α-solanine. Enzymes offer several advantages in food processing notably their specificity, ability to function under mild or harsh pH or temperature conditions and high activity. Furthermore, enzymes are considered natural products and therefore contribute towards the development of environmentally friendly and sustainable processes.

It has previously been established that both potato-derived extracts and extracts of fungal derived pathogens contain GA-degrading activities. Some of these enzymes appear to act by sequential degradation of the carbohydrate, indicating that multiple enzymes may be required for complete deglycosylation of the alkaloid. Furthermore, the enzymes that can degrade glycoalkaloids may be specific for only one of the two main GAs produced in potato, α-chaconine and α-solanine. It has been proposed that removal of the trisaccharide from α-chaconine and α-solanine would lead to solanidine and thereby detoxification of the compounds. Potato GAs consist of the solanidine aglycone with a carbohydrate side-chain, which is important for mediating interactions with cell membranes.

However, a recent study has shown solanidine had a greater impact on the physiology of the phytopathogen Phytophthora infestans compared to its glycosylated form. To date, only a handful of studies have documented the degradation of potato glycoalkaloids using microbial enzymes and to the best of our knowledge, no bacterial based studies exist.
We have recently identified a collection of bacterial strains capable of the complete degradation of GAs\(^8\). In this study, we report the discovery of a gene cluster for the deglycosylation of the glycoalkaloids \(\alpha\)-chaconine and \(\alpha\)-solanine from the soil bacterium *Arthrobacter* sp. S41.

2. **Materials and Methods**

2.1 **Strains and culture conditions**

Bacterial strains were routinely cultured in liquid or solid (1.5% agar) Lysogeny Broth (LB) medium at 20°C (*Arthrobacter* sp. S41) or 37°C (*Escherichia coli* for cloning and expression). When required, the medium was supplemented with 100\(\mu\)g ml\(^{-1}\) ampicillin.

2.2 **Data availability**

*Arthrobacter* sp. S41 was isolated in a previous study\(^8\). The draft genome sequence is available from Genbank, acc. no. SIHY01000000\(^15\).

2.3 **Sequence analysis**

Predicted protein sequences were compared with homologs at NCBI using BLASTp searches against the PDB and nr databases. Multiple alignment and the phylogenetic tree were constructed using MEGA6\(^16\). The integrated ClustalW algorithm was used for aligning GH2, GH3 and GH78 protein sequences. The alignment was manually inspected and then used to construct an unrooted Neighbor-Joining (NJ) phylogenetic tree based on 1,000 bootstrap replicates.

2.4 **Gene cloning and production of recombinant proteins**

Plasmid pET15b (Novagen) harbouring the ampicillin selectable marker and an N-terminal HIS tag was modified to contain a USER\(^\text{TM}\) cassette\(^17\) and subsequently used as a cloning and heterologous expression vector. A gene cluster was amplified from *Arthrobacter* sp. S41 genomic DNA by PCR.
using Pfu-X7 polymerase\textsuperscript{18} kindly provided by Prof. Halkier at the University of Copenhagen, using the forward (5’-ACGGATCU GTGATGACCGAGTCCAGTTC -3’) and reverse (5’-AGCCGGAU TCATGCTTGGATCGAT TC-3’) primers to yield a 7,582 bp fragment, which was fused by USER\textsuperscript{TM} cloning\textsuperscript{19} into pET15b linearised by PCR as described previously\textsuperscript{17}. The USER ligation mixture was transformed into E. coli TOP10 cells and incubated overnight at 37°C on LB agar supplemented with 100 \(\mu\)g ml\(^{-1}\) ampicillin. Recombinant colonies were picked from plates and cultivated in LB both supplemented with 100 \(\mu\)g ml\(^{-1}\) ampicillin at 37°C overnight with shaking. Recombinant plasmids were purified using the MiniPrep Plasmid DNA Purification Kit (Qiagen) and fusion of the amplicon and plasmid was confirmed by restriction digest of plasmid DNA followed by Sanger sequencing (GATC-Biotech, Konstanz, Germany) to confirm integrity of the DNA sequence. The resultant construct was then transformed into E. coli BL21 (DE3) \(\Delta\)lacZ competent cells as described above. For enzyme production, E. coli BL21(DE3) \(\Delta\)lacZ harbouring either pET22b (control) or pET15b_gene cluster (tester) were cultivated in 20 mL ZYP-5052 autoinduction medium\textsuperscript{20} supplemented with ampicillin (100 \(\mu\)g ml\(^{-1}\)) for 3 days at 20°C with shaking. Cells were collected by centrifugation and resuspended in 2 mL lysis buffer (50 mM HEPES, pH = 7.5). Cell lysis was performed in a FastPrep-24\textsuperscript{TM} 5G bead beater (MP Biomedicals) using glass beads size 212–300 \(\mu\)m/425–600 \(\mu\)m in the ratio 1:1 (Sigma-Aldrich). After cell disruption, the soluble and insoluble protein fractions were separated by centrifugation.

2.5 Enzyme reactions and activity visualization

Crude recombinant cell lysates were screened for activity against 4-Nitrophenyl \(\alpha\)-L-rhamnopyranoside (\(pNP\)) glycosides: \(pNP\)-\(\alpha\)-L-rhamnopyranoside, \(pNP\)-\(\beta\)-D-galactopyranoside, and \(pNP\)-\(\beta\)-D-glucopyranoside supplied by Sigma-Aldrich (Brøndby, Denmark) and against the potato GAs \(\alpha\)-chaconine and \(\alpha\)-solanine supplied by PhytoLabs (Vestenbergsgreuth, Germany). For testing enzyme
activity against 1 mM pNP-glycosides, 5 µl crude enzyme extract was added to 95 µl HEPES buffer (pH 7.5) and incubated at 25°C for 1 hour and absorbance at 405 nm was measured using EPOCH™ Microplate Spectrometer. For testing against GA substrates, enzymatic reactions were prepared and analyzed by thin layer chromatography (TLC) as described previously.

2.6 Liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) analysis of glycoalkaloids

Glycoalkaloids was extracted from the samples as previously described with modifications. Briefly, 10 µL of samples was added with 990 µL 5 % acetic acid with 0.3 µg/mL tomatine (internal standard). Tomatine was used to correct for variation in the extract of glycoalkaloids from samples but is not naturally present in raw potato fruit juice (RPFJ). Samples were shaken for 15 min followed by centrifugation (15 min, 14,000 x g at 4°C). The supernatant was collected for purification using solid phase extraction (HLB Oasis 1cc 30mg, GE Healthcare) on a vacuum manifold. The column was preconditioned with methanol (1 mL, three times) followed by equilibration with milliQ water (1 mL, three times) before the samples was added (1 mL). The column was washed with 10% methanol (1 mL, three times) before the glycoalkaloids were eluted with 1 mL methanol containing 0.1% formic acid. The eluate was filtered through a 0.2 µm Mini-UniPrep syringeless filter (Whatman, Maidstone, United Kingdom). The liquid chromatography separation was performed as described previously on an Agilent LC 1100 series HPLC system with an electrospray source. Ten µL of each sample was loaded onto Kinetex C18 column (250 × 4.6 mm) with a particle size of 5 µm (Phenomenex, Torrance, CA, USA). The LC eluent was initially set to 72% solvent A (0.1% formic acid) and (28% B solvent B (99.9% acetonitrile, 0.1% formic acid) and with a flow rate of 500 µL/min. Then, the LC gradient increased to 42% B over 11 mins and then to 41% B over 1 min and to 45% B over 8 min. Subsequently, the LC gradient was increased to 90% B over 1 min and kept at 90% B for 5 min before
returning to 28% B over 1 min. Each sample run was followed by a 32-min column wash. The mass spectrometry analysis was carried out on a single-quad mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). Electrospray ionization was performed in positive mode. Quantification was conducted in selected ion monitoring mode. Ions for α-solanine were recorded as \( m/z \) 868.4, 560.4 and 398.2, ions selected for α-chaconine were \( m/z \) 852.4, 560.4 and 398.2, for solanidine the ion was \( m/z \) 398.2, and for tomatine \( m/z \) 1034.4, 578.4 and 416.2. Quantification was done on all ions for each compound. The concentration of each glycoalkaloid was calculated as the integrated intensity of each target ion and compared to an external standard curve of α-solanine (0.03 µg/mL to 3 µg/mL), α-chaconine (0.03 µg/mL to 3 µg/mL) and solanidine (0.03 µg/mL to 1 µg/mL).

2.7 Enzymatic treatment of raw potato fruit juice (RPFJ)

RPFJ was obtained from Karup Kartoffelsmelsfabrik (Karup, Denmark). The RPFJ was analysed and found to have pH 5.94 and conductivity of 11.51 mS/cm. A 100 ml of RPFJ was treated with either no enzyme extract, 10 µl, 20 µl, 50 µl or 100 µl enzyme extract and incubated for 1 hour at 25°C. Samples were then heated to 100°C to stop enzyme activity. A 50 ml aliquot was then freeze dried for LC-ESI/MS analysis.

3. Results and Discussion

In a previous study, several morphologically distinct bacterial strains were obtained from enrichment cultures using minimal media containing either α-chaconine or α-solanine\(^8\). Among these isolates, strain S41 was selected for further detailed analyses because of its ability to grow on potato GAs. The isolated strain was identified as Arthrobacter sp. based on 16S rDNA analysis\(^8\). Arthrobacter sp. S41 was capable of growth on both α-chaconine and α-solanine as a sole carbon source suggesting that the
isolate could degrade both compounds. Removal of the carbohydrate moiety is the first step in the multistep biodegradation of α-chaconine or α-solanine. It has been suggested that removal of the trisaccharide detoxifies the GAs to release solanidine.

To investigate the possibility of removal of the carbohydrate moiety, we investigated the potential for the strain to produce GA-degrading enzymes. To identify a deglycosylation gene cluster in *Arthrobacter* sp. S41, the genome was sequenced and based on structural analysis of the compounds mined for α-rhamnosidases, β-glucosidases and β-galactosidases, predicted to be involved in deglycosylation. Sequencing and genome mining revealed a gene cluster containing three open reading frames (ORFs) putatively encoding an α-rhamnosidase (*rhaA*), a β-glucosidase (*gluA*) and a β-galactosidase (*galA*) clustered in a contiguous genomic region spanning approximately 7,582 bp (*Figure 1A*). As shown in *Table 1*, the putative products of the deglycosylation gene cluster show a high similarity to genes found in *Microbacterium azadirachtae* isolated from the rhizoplane of neem seedlings\(^23\). The organization of the gene cluster in the S41 strain was almost identical with that of the gene cluster identified in *M. azadirachtae*, with respect to sequence and position of the enzyme-encoding genes. A Shine-Dalgarno sequence, AGGAGC, was identified 9 bp upstream of the start codon of ORF2728 (*rhaA*) (*Figure 1B*). A predicted promoter sequence was identified with a -10 box and -35 box located 94 bp and 121 bp respectively upstream of ORF2728 start codon (*Figure 1B*).

Several ORFs (2725-2722), were identified downstream of ORF2726, *galA*. Only ORF2725 was also found in *M. azadirachtae*. ORF2724 showed 97% protein identity to a divalent metal cation transporter found in *Arthrobacter* sp. PA019 isolated from maize rhizosphere soil (24) (*Table 1*). ORF2723
showed 95% protein identity to a putative hydro-lyase from *Glutamicibacter halophytocola* and ORF2722 showed 95% similarity to an adenosine deaminase also from *G. halophytocola* (Table 1).

To examine the function of the enzyme-encoding genes, the complete gene cluster was cloned into *E. coli*. Cell lysates expressing the GA-degrading genes were tested with various pNP-glycosides which confirmed the functions of all three enzymes as an α-rhamnosidase, a β-glucosidase and a β-galactosidase (Figure 3A) substrates to confirm the function of all three enzymes. *E. coli* cell lysates were subsequently incubated with α-chaconine and α-solanine and enzymatic degradation was visualized by TLC (Figure 3B). TLC analysis showed that the gene cluster degraded both α-chaconine and α-solanine to release solanidine. LC-ESI/MS analysis indicated high enzyme activity with the removal of both α-chaconine and α-solanine after 1 hour incubation with the substrates (Figure 4). The main hydrolysis product was solanidine confirming the role of the gene cluster in the removal of the carbohydrate moiety of GAs.

The potential application of the enzymes in food processing was investigated by testing activity on RPFJ. RPFJ was treated with different concentrations of *E. coli* cell lysates expressing the deglycosylation gene cluster and incubated 1 hour. Addition of 20 µl of enzyme extract significantly reduced the total GAs present in samples (Figure 5, Table 2). Using an enzyme concentration 5-fold higher resulted in complete removal of both α-chaconine and α-solanine after 1 hour (Figure 5, Table 2) as previously observed against pure GA substrates. It was observed that α-solanine was not as easily hydrolyzed as α-chaconine possible indicating the presence of inhibitors in the RPFJ that may affect enzyme activity. However, upon preparation of the enzyme extract, it was observed that the β-
galactosidase was not as well expressed in the prepared batch when tested on pNP substrates in comparison to the other three enzymes (data not shown).

Based on the LC-ESI/MS analysis it would appear that two monosaccharides from both α-chaconine and α-solanine are removed producing the intermediate compounds γ-chaconine and γ-solanine respectively (Figure 6). In order to release this intermediate product two enzyme activities are required indicating that a third enzyme (either β-galactosidase or β-glucosidase) is then required to remove the final monosaccharide to release solanidine. Further work will be carried out to evaluate the role of each enzyme alone or in combination to determine the different steps involved in the degradation of both compounds.

The results of this study reveal a new gene cluster harbouring novel enzymes for the deglycosylation of toxic glycoalkaloids from potato. To the best of our knowledge, this is the first report describing bacterial enzymes involved in the hydrolysis of both α-chaconine and α-solanine. Based on a previous study, the native isolate Arthrobacter sp. S41 is capable of metabolizing solanidine and therefore there remain novel genes with functions still unknown that may be involved in the complete degradation of potato GAs. No reports currently exist on the microbial enzymatic degradation of solanidine. This suggests the involvement of novel enzymes and hitherto uncharacterized pathways for GA degradation and potentially other plant secondary metabolites.

ACKNOWLEDGEMENTS
E. coli strain BL21ΔlacZ kindly provided by Professor Jin-Ho Seo, Seoul National University, South Korea. This work was supported by the Innovation Fund Denmark grant 5158-00001A (proPotato: Potato Proteins – Challenges and Industrial Possibilities).
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Legends to figures

Figure 1 Genetic organization of the deglycosylation gene cluster from *Arthrobacter* sp. S41. Genes are indicated by arrows orientated in the direction of transcription (A). Putative promoter sequence: Shine-Dalgarno sequence (SD) and -10 and -35 boxes located upstream of the START codon of *rhaA* (B).

Figure 2 Sequence-based relationship of GH2, GH3 and GH78 family proteins from *Arthrobacter* sp. S41 and homologs obtained from the CAZy database. The phylogenetic tree was constructed using the Neighbor-Joining method and bootstrap analysis (1000 replicates) of a ClustalW alignment of amino acid protein sequencing using MEGA 7. Accession number and taxonomy is indicated for each protein. Triangles represent characterized enzymes according to the CAZy database and circles represent putative enzymes.

Figure 3 Recombinant expression and activity of glycoalkaloid (GA) degrading gene cluster from *Arthrobacter* sp. S41. *Escherichia coli* cell lysates were screened for activity against against pNP-glycosides pNP-α-L-rhamnopyranoside (blue), pNP-β-D-galactopyranoside (green) and pNP-β-D-glucopyranoside (purple) (A). Thin layer chromatography (TLC) was used to analysis hydrolysis by *Escherichia coli* BL21 ΔlacZ cell lysates expressing the empty vector pET15b (C; control) and the GA-degrading gene cluster isolated from *Arthrobacter* sp. S41 (T; tester) compared to pure GAs (S; standard) and solanidine (B).

Figure 4 Analysis of glycoalkaloid (GA) degradation using liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) operated in selected ion monitoring mode. Total ion
chromatogram of glycoalkaloids identified in the degradation of α-chaconine and α-solanine by
Escherichia coli BL21 ΔlacZ cell lysates expressing the empty vector pET15b (C; control) (B) or the
GA-degrading gene cluster isolated from *Arthrobacter* sp. S41 (T; tester) (C) compared to GA
standards (S) (A).

Figure 5 Liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) analysis
of raw potato fruit juice (RPFJ). *Escherichia coli* BL21 ΔlacZ cell lysates expressing the GA-
degrading gene cluster isolated from *Arthrobacter* sp. S41 was added to 100 ml RPFJ and total GA
concentrations quantified.

Figure 6 Proposed pathways for the degradation of α-chaconine and α-solanine by *Arthrobacter*
sp. S41.

Table 1 *In silico* analysis of the *Arthrobacter* sp. S41 glycoalkaloid (GA) degrading cluster and
neighboring genes

Table 2 Liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS)
analysis of raw potato fruit juice (RPFJ). RPFJ was treated for 1 hour with *Escherichia coli*
BL21ΔlacZ cell lysates expressing the empty vector pET15b (Control) or the GA-degrading gene
cluster isolated from *Arthrobacter* sp. S41 (Tester).
Figure 1

A

B

-35 box
TTTTGATGGATTGACTTTGTGCGGCCGTAGCTCACGTTGTTATGTTGAGCCGTAACCTAG

SD
CTCATCTGTAAGGACCGGCGGTCAAGCTGATGACCGAGTCCAGTTCTGCGCSTTTCG

START
CTCACCTCCTTTTGCGCAGGGAGTCTGTTGCTG

-10 box
CTCGCCGCTAGTTGAATTTCTGACAACGGAATCTCGGTAGGCGGACTATCGGAAGCT

SD
CTCATCTGTAAGGACCGGCGGTCAAGCTGATGACCGAGTCCAGTTCTGCGCSTTTCG

START
CTCACCTCCTTTTGCGCAGGGAGTCTGTTGCTG
Figure 3

A

B

ACS Paragon Plus Environment
Figure 4

A

Retention time (min)

Time = 0h

Time = 1h

Time = 24h

Peak intensity

α-solanine

α-chaconine

B

Retention time (min)

Time = 0h

Time = 1h

Time = 24h

Peak intensity

α-solanine

α-chaconine

C

Retention time (min)

Time = 0h

Time = 1h

Time = 24h

Peak intensity

560 m/z

solanidine
Figure 5

Retention time (min) vs. Ion intensity for different extract volumes (10 µL, 20 µL, 50 µL, 100 µL) and control 1 h. Peaks for α-solanine, α-chaconine, m/z 560, m/z 702, and solanidine are highlighted.
Figure 6

α-solanine (868 m/z)

γ-chaconine/γ-solanine 560 m/z

Solanidine 398 m/z

α-chaconine (852 m/z)
Table 1 *In silico* analysis of the *Arthrobacter* sp. S41 glycoalkaloid (GA) degrading cluster and neighboring genes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Closest Relative</th>
<th>Accession Number</th>
<th>Percentage ID (%)</th>
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<td>2730</td>
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<td>2729</td>
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<td>adenosine deaminase, <em>G. halophytocola</em></td>
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Table 2 Liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) analysis of raw potato fruit juice (RPFJ). RPFJ was treated for 1 hour with *Escherichia coli* BL21ΔlacZ cell lysates expressing the empty vector pET15b (Control) or the GA-degrading gene cluster isolated from *Arthrobacter* sp. S41 (Tester).

<table>
<thead>
<tr>
<th>Samplea</th>
<th>Volume added (µl)b</th>
<th>α-Chaconinec</th>
<th>α-Solaniec</th>
<th>Solanidinec</th>
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</thead>
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<tr>
<td>RPFJ</td>
<td>-</td>
<td>2172.1 ± 185.6</td>
<td>2127.5 ± 172.2</td>
<td>11.0 ± 1.9</td>
</tr>
<tr>
<td>RPFJ heated</td>
<td>-</td>
<td>2135.0 ± 136.8</td>
<td>2186.1 ± 102.4</td>
<td>17.6 ± 1.6</td>
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<tr>
<td>Control</td>
<td>10</td>
<td>1967.6 ± 102.2</td>
<td>2089.4 ± 106.3</td>
<td>16.4 ± 1.0</td>
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<tr>
<td>Control</td>
<td>20</td>
<td>1914.5 ± 164.4</td>
<td>2001.0 ± 227.9</td>
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<tr>
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<td>50</td>
<td>1818.8 ± 154.6</td>
<td>1935.9 ± 152.5</td>
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<td>77.0 ± 8.5</td>
<td>1599.5 ± 58.5</td>
<td>584.1 ± 6.8</td>
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</table>

*aRPFJ non treated, heated or treated with control or tester and heated before LC-ESI analysis

bVolume of *E. coli* cell lysate extract added per 100 ml of RPFJ

cConcentration of α-chaconine, α-solanine, and solanidine (µg per g of potato)