CHARACTERIZATION OF A NITROREDUCTASE WITH SELECTIVE NITROREDUCTION PROPERTIES IN THE FOOD- AND INTESTINAL-OCCURRING LACTIC ACID BACTERIUM LACTOBACILLUS PLANTARUM WCFS1

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Running title: A selective nitroreductase in *Lactobacillus plantarum*

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SUMMARY

Nitroreductases reduce nitroaromatic compounds and other oxidants in living organisms having interesting implications in environmental and human health. A putative nitrobenzoate reductase encoding gen (lp_0050) was recently annotated in the completed DNA sequence of lactic acid bacterium *Lactobacillus plantarum* WCFS1 strain. In this research, this *L. plantarum* gen was cloned, expressed and the corresponding protein (PnbA) biochemically characterized. This *L. plantarum* PnbA reductase is a 216 amino acid residues FMN-flavoprotein which exhibits a 23 % identity with *Pseudomonas putida* and *Ralpstonia euthropha* nitroreductases, and less than 11 % with those from enterobacteria such as *E. cloacae*. This reductase also showed 32-43% identity (65-72 % similarity) to predicted PnbA proteins from other lactic acid bacteria. It utilized a wide range of electron acceptors including dichlorophenolindophenol (DCPIP), nitroblue tetrazolium (NBT), ferricyanide and quinones (menadione, benzoquinone), but not pydinium cations (paraquat and N-methyl-β-carbolinium), and it was inhibited by dicoumarol and diphenyliodonium. HPLC-MS and spectroscopic data showed that it specifically catalyzed the reduction of 4-nitroaromatic group to the corresponding hydroxylamino in the presence of NAD(P)H. Kinetics parameters (V_{max} and K_m) showed a much higher efficiency to reduce 2,4-dinitrobenzoate than 4-nitrobenzoate. It was chemoselective for reduction of 4-nitrobenzoates being unable to reduce other nitroaromatics. Then, *L. plantarum* PnbA reductase and perhaps similar reductases in other lactic bacteria might be more specific than other microbial nitroreductases which reduce a wider range of nitroaromatic compounds. The physiological and functional role of nitroreductases remain unknown, however their presence in

lactic acid bacteria widely occurring in foods and the human intestinal tract should be of further interest.

INTRODUCTION.

Lactic acid bacteria are common in foods and the mammalian intestine. Among them, *Lactobacillus plantarum* is a versatile lactic acid bacterium encountered in a range of environmental niches that has a proven ability to survive and can colonize the intestinal tract of human and other mammals (12). The complete genome sequence of the *L. plantarum* WCFS1 has become recently available (24), making this bacteria a suitable model to explore the molecular mechanisms underlying the targeted intestinal properties of this species (12). From its complete genome sequence analysis, a sequence (lp_0050) putatively encoding a protein annotated as *p*-nitrobenzoate reductase (PnbA) was found, although it has not been further produced and/or characterized. Nitroreductases usually catalyze the reduction of nitrocompounds that are cytotoxic and mutagenic (2, 38). Despite their toxicity, some bacteria have been found to harbor enzymes for the metabolism of nitroaromatics, and they have been considered of great potential application in environmental bioremediation, biocatalysis, and as chemotherapeutic agents following the bioactivation of prodrugs (4, 45, 47, 52, 54, 55, 57).

Nitroreductases belong to two main classes: oxygen-insensitive (type I) and oxygen-sensitive (type II) (25). The former abounds in bacteria and fungus (11), and the latter in mammalian systems. Oxygen-sensitive nitroreductases usually catalyze one-electron reduction yielding a nitro anion radical (42) that reacts with oxygen to form superoxide radical regenerating the nitro group. In

this “futile cycle” pyridine nucleotides are oxidized without net reduction of the nitro group. In absence of oxygen further reduction of the nitroaromatic may yield nitroso, hydroxylamine and amino derivatives (42). Oxygen-insensitive nitroreductases are a family of bacterial FMN-containing enzymes that catalyzes the reduction of nitro compounds producing nitroso, hydroxylamino and/or amino derivatives (47, 49, 52). These nitroreductases have been studied in a number of bacteria including enterobacteria (*e.g. E. coli, S. typhymurium, E. cloacae*) , *Pseudomonas*, and *Rhodobacter capsulatus* (5, 6, 19, 41, 44, 46, 53, 58), and exhibit varying substrate specificity being able to reduce a wide range of nitroaromatics such as nitrophenols, nitrobenzenes and nitrobenzoates. So far, the physiological and functional role of microbial nitroreductases remain unknown (47), but it may include: a possible role in quinone (or oxidants) reduction and detoxification (46), oxidative stress (32, 33), bioluminiscence (28), cobalamin synthesis (43), or even a function involving specific degradation pathways of nitrocompounds (20, 45, 47).

A large number of nitroaromatics are present in the environment because of their use in manufacturing processes, as antimicrobial agents or generated as by-products of combustion processes. Biodegradation of these compounds by using selected microorganisms and nitroreductases can be of great interest in biodegradation of environmental pollutants (7, 45, 47). Nitroaromatic compounds have also attracted a considerable health concern since their metabolization through reductive pathways may lead to formation of potent genotoxic and/or mutagenic metabolites (30, 34, 38). Indeed, nitro compounds are able to generate reactive nitrogen oxide species which readily react with biological molecules (50). Although the metabolic pathways of these

compounds are complex, nitroreductases appear to have a central role in their bioactivation (11, 14). Thus, enzymatic reduction by nitroreductases gives rise to reactive intermediates that can undergo nucleophilic additions with DNA and other macromolecules, suggesting a possible mechanism for their cytotoxicity (2).

Lactic acid bacteria occurring in mammalian intestinal microbiota and fermented foods might metabolize nitroaromatics having possible environmental, health and toxicological implications (12, 44). In this regard, the goal of this work was to study the biochemical and functional characteristics of a novel Pnb reductase from the lactic acid bacterium, *Lactobacillus plantarum* WCFS1. The gene encoding the PnbA reductase from this bacterium was cloned and the recombinant protein overexpressed in *Escherichia coli*. This functional nitroreductase was a FMN flavoenzyme able to reduce several electron acceptors, including various quinones but not pyridinum cations, and it was selective for the reduction of specific 4-nitroaromatic compounds, exhibiting a potent reduction for 4-nitrobenzoate and 2,4-dinitrobenzoate to 4-hydroxylamine metabolites. Then, this nitroreductase presents in lactic acid bacteria that occur both in foods and intestinal human microflora might play a role in the metabolism and reduction of nitroaromatics and oxidants.

MATERIAL AND METHODS

Chemicals and reagents. FMN, NADPH, NADH dicoumarol, diphenyliodonium chloride (DPI), dichlorophenolindophenol (DCPIP), ferricyanide, nitroblue tetrazolium (NBT), cytochrome C, menadione (2-methyl-1,4-naphtoquinone), 1,4-benzoquinone, p-nitrophenyl-β(D)-glucopyranoside and 1,1'-dimethyl-4,4'-

126 bipyridinium dichloride hydrate, 4-nitrobenzoic acid, 3-nitrobenzoic acid, 2-
127 nitrobenzoic acid, 2,4-dinitrophenol, 5-nitroindazole and 2-amino-4-nitrophenol
128 were obtained from Sigma-Aldrich, 2,4-dinitrobenzoic acid and nitrobenzene
129 were from Fluka, 3,5-dinitrosalicilic acid from Merck, and picric acid from
130 Probus. *N*-methyl-β-carbolinium iodide (2-methyl-9*H*-pyrido-(3,4-*b*)-indole) (*i.e.*
131 2-methylnorharmanium and 2-methylharmanium) were synthetized from
132 norharman and harman with methyl iodide in acetone (16, 17). Several
133 nitroderivatives such as *N*-(2-nitrophenyl)-*L*-proline, *N*-(2-nitrophenyl)pipecolic
134 acid, *N*-(2,4-dinitrophenyl)glycine, *N*-(2-nitrophenyl)glycine were obtained as
135 previously (9). p-Hydroxylaminobenzoic acid was synthesized from 4-
136 nitrobenzoic acid by reduction with Zn in presence of $ClNH_4$, (3). For that, 17 g
137 of 4-nitrobenzoic acid were dissolved in water and sodium hydroxide. After
138 addition of 20 g of ammnonium chloride, the mixture was cooled and 15 g of
139 zinc dust was added gradually and stirring. After one-half hour stirring the zinc
140 sludge was filtered off. To the filtrate hydrochloric acid was added slowly and
141 amorphous flakes which separated while the reaction was still neutral, were
142 removed by filtration. Upon addition of more hydrochloric acid to the filtrate,
143 white needless separated of *p*-hydroxylaminobenzoic acid that was
144 characterized by spectral data. This compound was used for quantification
145 using the corresponding calibration curves.
146
147 **Bacterial strains, plasmids, and enzymes.** The *L. plantarum* strain that has
148 been completely sequenced, *L. plantarum* WCFS1 (NCIMB 8826), was used in
149 this study. *E. coli* DH5α was used for all DNA manipulations. *E. coli* JM109
150 (DE3) was used for expression in pURI3 vector (10). *L. plantarum* strain was

grown in MRS medium at 30 ºC without shaking. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 ºC and 200 rpm. When required, ampicillin or chloramphenicol were added to the medium at a concentration of 100 or 34 µg/mL, respectively. Chromosomal DNA, plasmid purification, and transformation of *E. coli* were carried out as described elsewhere (48).

Expression and purification of *L. plantarum* PnbA. The gene coding for the putative *p*-nitrobenzoate reductase, *pnbA* (lp_0050) from *L. plantarum* WCFS1 has been cloned and overexpressed in pURI3 vector to avoid the enzyme and ligation steps during the cloning (10). Expression vector pURI3 was constructed based on the commercial expression vector pT7-7 (USB) but containing a leader sequence with a six-histidine affinity tag. The *pnbA* gene was PCR amplified with Hot-start Turbo *Pfu* DNA polymerase by using the primers 321 (5´- *CATCATGGTGACGATGACGATAAG*atggaaacaattaaagcgattcaca) and 322 (5´- *AAGCTTAGTTAGCTATTATGCGTA*ttagttgataatatgcaaaacttgcgg) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *pnbA* gene sequence are written in lowercase letters). The 648 pb purified PCR product was inserted into the pURI3 vector by using a restriction enzyme- and ligation-free cloning strategy described previously (10). *E. coli* DH5α cells were transformed, recombinant plasmids were isolated and those containing the correct insert were identified by restriction-enzyme analysis, verified by DNA sequencing, and then transformed into *E. coli* JM109(DE3) (pLysS) cells for expression.

Cells carrying the recombinant plasmid, pURI3-pnbA, were grown at 37 ºC in Luria-Bertani media containing ampicillin (100 µg/ml) and chloramphenicol

(34 µg/ml), until they reach an optical density at 600 nm of 0.4 and induced by adding IPTG (0.4 mM final concentration). After induction, the cells were grown at 22 ºC during 20 h and collected by centrifugation. Cells were resuspended in 20 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl. Crude extracts were prepared by French press lysis of the cell suspension (three times at 1100 psi). The insoluble fraction of the lysate was removed by centrifugation at 47,000*g* for 30 min at 4 ºC.

The supernatant was filtered through a 0.45 µm filter and applied to a His-Trap-FF crude chelating affinity column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing imidazole, to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted by applying a stepwise gradient of imidazole concentration, from 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 10 mM imidazole to the same buffer but containing 500 mM imidazole. Fractions containing the His_6-tagged protein were pooled and dialysed overnight at 4 ºC against 10 mM Tris-HCl, pH 8.0, containing 10 mM NaCl in a membrane (3,500 cuttoff). The purity of the enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine buffer. Protein concentration was measured according to the method of Bradford using a protein assay kit (Bio-Rad) with bovine serum albumin as standard.

Reductase assays with electron acceptors. Activity of PnbA reductase was monitored using several electron acceptors as substrates as follows: a) Reduction of dichlorophenolindophenol (DCPIP) was assayed in a reaction mixture containing 100 mM phosphate buffer (pH 7.4), 500 µM NADPH, 200 µM DCPIP

and PnbA reductase (1-5 μg/ml protein) in a final volume of 0.5 ml. The reaction was initiated by the addition of substrate and the rate of DCPIP reduction with time was determined (λ= 600 nm; ε= 21.5 mM^{-1} cm^{-1}). Reaction rate was also calculated in absence of enzyme or NADPH as controls, and in presence of diphenyliodonium (0-10 mM) or dicoumarol (0-500 μM) used as inhibitors. Reduction of DCPIP was also used to determinate the activity in presence of NADH (500 μM) replacing NADPH as electron donor and to study the optimal temperature of reduction after incubating the enzyme for 20 min at different temperatures; b) Reduction of cytochrome C in presence or absence of menadione as an electron acceptor was measured using a reaction mixture containing 90 mM Tris-HCl buffer (pH 7.4), 500 μM NADPH, menadione (0 or 20 μM), 0.96 mg/ml of cytochrome C and reductase (1-5 μg/ml protein) in a final volume of 0.5 ml. The activity of reductase was started by addition of NADPH and the reduction rate of cytochrome C at 550 nm was measured (ε= 21 mM^{-1} cm^{-1}); c) Reduction of ferricyanide was measured by using a mixture containing 90 mM phosphate buffer (pH 7.4), 500 μM NADPH, 1mM potassium ferricyanide ($k_3[Fe(CN)_6]$ and reductase enzyme (1-5 μg/ml protein). Reduction of ferricyanide was measured at 420 nm (ε= 1020 M^{-1} cm^{-1}). This assay was also used for determination of optimal pH using buffers adjusted at pHs between 2 and 9; d) Reduction of nitroblue tetrazolium (NBT) was monitored in a reaction mixture containing 80 mM phosphate buffer (pH 7.4), 500 μM NADPH, 200 μM NBT and enzyme (1-5 μg/m protein) in a final volume of 0.5 ml. The reaction was started with the addition of substrate and monitored with time at 580 nm (ε= 7 mM^{-1} cm^{-1}).

Reduction of nitroaromatics, quinones and pyridinium cations by *L. plantarum* PnbA reductase. The activity of recombinant PnbA reductase from *L. plantarum* was assayed with different substrates, including a number of nitrocompounds, pyridinium cations and quinones by two different methods: HPLC-DAD-mass spectrometry and by following the spectrophotometric NADPH depletion. Thus, reaction mixtures with a final volume of 0.5 ml containing reductase enzyme (usually from 0.25 to 5 μg/ml protein) , NADPH (500 μM) and the corresponding substrate (from 0 to 1 mM) in 100 mM buffer phosphate pH 7.4 were mixed and incubated at room temperature for 3 min; the reaction stopped with the addition of 10% 2N HCl, and subsequently injected into HPLC and HPLC-MS to quantify and identify metabolites of *L. plantarum* reductase and to calculate the reaction rates. 4-Nitrobenzoic acid, 2,4-dinitrobenzoic acid, 2-nitrobenzoic acid, 4-hidroxylaminobenzoic acid, 2,4-dinitrophenol and 3-nitrobenzoic acid were tested as substrates for PnbA reductase. The corresponding controls without reductase and/or NADPH were also carried out. Kinetic values of K_m and V_{max} were determined by non-linear regression analysis fitting to Michaelis-Menten curves of reaction rates of *p*-hidroxylamino products as a function of the concentration of nitroaromatic substrates from 0 to 1mM. Enzyme reaction rate was calculated as μmoles of hydroxyliamino product/min mg of protein. On the other hand, the activity of *L. plantarum* PnbA reductase was also measured by monitoring the decrease in absorbance of NADPH at 340 nm in a spectrophotometer for two 2-6 min in presence of various nitrocompounds or quinones as substrates. For that, reaction mixtures (0.5 ml) containing reductase enzyme (from 1 to 5 μg/ml), NADPH (300 μM) and substrate (50 or 100 μM) in 100 mM phosphate buffer (pH 7.4) were added to quartz cuvettes. The reaction was

started by the addition of substrates and the reduction rate was calculated as the μmoles of NADPH oxidized/min mg of protein.

Reversed phase HPLC chromatographic analysis. The analysis of enzymatic reaction media was performed by RP-HPLC with UV diode array and fluorescence detection using a HPLC 1050 (Hewlett Packard) with a 1100 diode array detector (DAD) and a 1046A-fluorescence detector. A 150 mm x 3.9 mm i.d., 4 μm, Nova-pak C18 column (Waters, Milford, MA, USA) was used for chromatographic separation. Chromatographic conditions were: 50 mM ammonium phosphate buffer (pH 3) (buffer A) and 20% of A in acetonitrile (buffer B). The gradient was programmed from 0% (100% A) to 32% B in 12 min, and 90% B at 15 min. The flow rate was 1 ml/min, the column temperature was 40 ºC and the injection volume was 20 μl. Absorbance detection was set at 280 nm (analysis of 4-hydroxylaminobenzoic acid) and 243 nm (analysis of 2-nitro-4-hidroxylaminobenzoic acid). Identification of compounds was performed by UV spectra, mass spectrometry and coinjection with synthetic standards. For quantification of metabolites, a calibration curve of synthetized 4-hidroxylaminobenzoic acid was constructed (0 to 50 μM) whereas for quantification of 2-nitro-4-hidroxylaminobenzoic acid, the response factor at 243 nm of a calibration curve of 2,4-dinitrobenzoic acid was used because both substrate and product exhibited similar spectra.

Identification by HPLC-ESI-mass spectrometry. To carry out mass spectrometric identification, reactions media of *L. plantarum* PnbA reductase, nitroaromatics and NADPH obtained as above were analyzed on a 150 X 2.1 mm *i.d.* Zorbax SB-C18, 5 μm, column (Agilent Technologies) by using a series 1100

HPLC-MSD (Hewlett-Packard) (electrospray-positive and negative ion mode). Eluent A: acetic acid (0.5%); B: 20 % of A in acetonitrile. Gradient: 100 % A at 0 min and 60 % B at 20 min and 100 % B at 25 min, flow rate 0.25 ml/min; T: 40 ºC; mass range 50-500 *amu*, flow gas 11 l/min and Tª 330 ºC, and cone voltage 55 V. Pressure of nebulizador 40 psi and capilar voltage 4000 V.

Spectrophotometric studies. UV-VIS spectra were recorded with a Beckman spectrophotometer. Samples with the purified reductase from *L. plantarum* were placed in quartz cuvettes and spectra were determined in anaerobic conditions, before and after the addition of an excess of sodium dithionite ($Na_2S_2O_3$). The same apparatus was used for the determination of enzyme activities based on the decrease of NADPH (340 nm) in presence of substrate at room temperature as indicated above.

RESULTS

Cloning, expression and purification of L. plantarum PnbA. The deduced product of *L. plantarum pnbA* is a protein of 216 amino acid residues, 23.9 kDa, and pI of 6.65. This protein is included in the nitroreductase Pfam family (PF00881) that contains proteins involved in the reduction of nitrogen compounds (http://pfam.sanger.ac.uk/family?acc=PF00881). BLAST databases searches of the translated *L. plantarum* DNA sequence showed high-scoring similarities (32-43% identity) with putative *p*-nitrobenzoate reductases (PnbA) from the complete sequenced lactic acid bacteria (data not shown), suggesting that these enzymes may exhibit similar functional properties in different lactic acid bacteria. The predicted sequence of the *L.* plantarum PnbA was aligned

300 with selected nitrobenzoate reductases which biochemical activity has been
301 previously reported (Figure 1). From them, *L. plantarum* PnbA showed the
302 highest overall identity (23%) with that of *Pseudomonas putida* and *Ralstonia*
303 *euthropha*. *Enterobacter cloacae* PnbA showed only an 11% identity to *L.*
304 *plantarum* protein. Other nitroreductases from enterobacteria such as *E. coli*
305 and *S. typhymurium* also gave identities lower than 10 %. In contrast, identities
306 among nitroreductases from enterobacteria are higher than 80 % (59). As
307 deduced from Figure 1, all these proteins showed some of the residues
308 conserved in this family, belonging to the characteristic nitroreductase motif. A
309 2-nitroreductase enzyme from *Pseudomonas fluorescens* (20) not included in
310 the alignment was only 3-7% identical to the other nitroreductases, and it did
311 not present the conserved residues described for this family. In conclusion, on
312 the basis of its amino acid sequence, it could be assumed that PnbA (lp_0050)
313 from *L. plantarum* WCFS1 is a member of the nitroreductase family.
314
315 **Functional expression of L. plantarum PnbA.** To confirm that the *pnbA*
316 (lp_0050) gene from *L. plantarum* WCFS1 encodes a functional *p*-nitrobenzoate
317 reductase (PnbA), we expressed this gene in *E. coli* under the control of the T7
318 RNA polymerase-inducible Φ10 promoter. Cell extracts were used to detect the
319 presence of hyperproduced proteins by SDS-PAGE analysis. Control cells
320 containing the pURI3 vector plasmid alone did not show expression over the
321 time course analyzed, whereas expression of an additional protein was
322 apparent with cells harboring pURI3-pnbA (Figure 2). The molecular mass of
323 the recombinant protein corresponded to that inferred from the nucleotide
324 sequence (23.9 KDa). As the gene was cloned containing a purification poly-His
325 tag, PnbA was purified on a His-Trap- FF crude chelating column and eluted

with a stepwise gradient of imidazole. Highly purified PnbA protein was obtained from pURI3-pnbA (Figure 2) and fractions showing activity and usually eluting at 125 mM imidazole had yellow colour. The presence of the protein band in the gel of the elution fractions, correlated well with the occurrence of reductase activity using DCPIP (dichlorophenolindophenol) as electron acceptor in presence of NAD(P)H. The presence of the Histag had no apparent effect on catalytic activity. Thus, we could conclude that the *pnbA* (lp_0050) gene encoded a functional reductase in *L. plantarum* WCFS1. The eluted *L. plantarum* nitroreductase was dialyzed to eliminate the imidazole and the purified protein was used for its biochemical characterization.

The purified PnbA reductase from *L. plantarum* was yellow indicating the presence of a bound cofactor and displayed UV-visible absorption spectra characteristic of flavin-containing enzymes (Figure 3). Thus, spectra of the protein showed two bands (maxima at 366 and 446 nm) resembling an oxidized FMN cofactor which is usually present in bacterial nitroreductase whereas this profile quickly desappeared after reduction of the flavoenzyme with an excess of sodium dithionite as seen by the lost of absorbance at 446 nm and as expected for FMN enzymes. The enzyme appeared to lost part of the activity and colour with time suggesting that the coenzyme might not be tightly bound to the reductase (53). A further incubation of the protein with FMN appeared to partly recover activity (not shown).

Catalytic properties of PnbA reductase from L. plantarum. The catalytic properties of PnbA reductase from *L. plantarum* were studied with a number of electron acceptors in presence of NAD(P)H as electron donor (Table 1).

351 Nitroreductase from *L. plantarum* reduced DCPIP (absorbance at 600 nm) in
352 presence of NADPH. It reduced cytochrome C in presence of menadione,
353 whereas in the absence of menadione as an electron carrier and acceptor it
354 was a poor reductant (7.5 times lower) of cytochrome C, suggesting that this
355 enzyme was not a cytochrome P450-like reductase. Moreover, *L. plantarum*
356 PnbA reductase also reduced nitroblue tetrazolium (NBT) to give formazan and
357 was able to give electrons to the single electron acceptor ferricynanide.
358 Therefore, various types of substrates were electron acceptors of *L. plantarum*
359 PnbA reductase. As determined for the reduction of DCPIP, the enzyme could
360 use both NADPH and NADH as electron donors although using NADPH as
361 reductant was 1.8 times more efficient than NADH (500 μM). The optimal pH
362 was measured in the range of 2-9 and the best reduction rates (reduction of
363 ferricyanide) was achieved in the interval pH 5 and 7 (highest value around pH
364 6). The optimal temperature (reduction of DCPIP) was 25 ºC and the activity
365 highly decreased after incubating the enzyme at temperatures higher than 37
366 ºC for 20 min. Inhibition studies were carried out with dicoumarol and
367 diphenyliodonium, that are inhibitors of quinone reductases and flavoproteins.
368 Both compounds inhibited in a dose-dependent manner the PnbA reductase of
369 *L. plantarum*. Dicoumarol inhibited reduction of DCPIP with a IC_{50} of 0.15 mM
370 whereas diphenyliodonium (DPI) inhibited it with IC_{50} of 5.5 mM. Inhibition by
371 DPI was expected as it is an inhibitor of flavoproteins (8), whereas inhibition by
372 dicoumarol appears to need higher concentrations than those reported for
373 NADPH:quinone reductases (18).
374
375 As shown in Table 1**,** several electron acceptors of reductases were good
376 substrates of *L. plantarum* PnbA reductase in presence of NAD(P)H.

377 Subsequently, a number of nitroaromatics, quinones and naturally occurring
378 and toxic pyridinium cations were studied as substrates and electron acceptors
379 of this nitroreductase. The reduction rate was performed by monitoring the
380 decrease of NADPH in presence of the corresponding substrate (Table 2). The
381 pyridinium cations, paraquat and 2-methyl-β-carbolinium cations, were not
382 reduced significantly by PnbA reductase. However, the quinones, menadione
383 and benzoquinone, were good substrates of this enzyme with benzoquinone
384 showing five times more activity than menadione. Among a range of
385 nitroaromatic compounds containing nitro groups in different positions in the
386 benzenic ring, including nitrophenols, nitrobenzene and nitrobenzoic acids, only
387 two nitro compounds: 4-nitrobenzoate and 2,4-dinitrobenzoate were substrates
388 of this enzyme as showed by an increased removal of NADPH (340 nm) when
389 compared with a control in absence of the nitroaromatic substrate. The activity
390 for reduction of 2,4-dinitrobenzoic acid was 12-fold higher than for 4-
391 nitrobenzoate, suggesting that the former was a better substrate for *L.*
392 *plantarum* PnbA reductase. From these results only specific nitro compounds
393 could be reduced in presence of NADPH by PnbA reductase.
394
395 **Identification of metabolites in the reaction mixtures by HPLC-MS.** In order
396 to characterize the reduction products of nitrocompounds, enzymatic reaction
397 media containing *p*-nitrobenzoic acid, NADPH and *L. plantarum* PnbA
398 reductase were studied by HPLC-DAD and MS (electrospray) (Figure 4). p-
399 Hydroxylaminobenzoic acid was identified as a major metabolite in the reaction
400 media containing *p*-nitrobenzoic acid as shown by HPLC-MS, with ions at *m/z*
401 154 $(M+H)^+$, 137 (M+1-17) (positivie-ESI) and *m/z* 152 $(M-H)^-$ (negative-ESI)

(Figure 4a). This metabolite coeluted with a synthetic standard of *p*-hydroxylaminobenzoic acid and afforded similar absorption spectra (*max.* at 278 nm). In the reaction mixtures, a peak of *N*-nitrosobenzoic acid (negative-ESI) at *m/z* 150 $(M-H)^-$ also appeared, suggesting that it could be an intermediate in the reaction. In addition, traces of *p*-aminobenzoic acid eluting after the corresponding *p*-hydroxylamine compound (*m/z* at 136 $(M-H)^-$ and 138 $(M+H)^+$ in negative and positive ion mode) were detected. This amine product was not increased in the reaction media suggesting that it was not a final product of PnbA reductase, and it might have appeared from chemical reduction. Indeed, no reduction was observed when synthetic *p*-hydroxylaminobenzoic acid was used as substrate of PnbA reductase in presence of NADPH. On the other hand, 2,4-dinitrobenzoic acid was also reduced in the reaction media by *L. plantarum* PnbA reductase and a major metabolite was identified by HPLC-MS (Figure 4b) as *p*-hydroxylamino-2-nitrobenzoic acid in ESI-positive at *m/z* 199 $(M+H)^+$ and 181, and ESI-negative at *m/z* 197 $(M-H)^-$ and 395 $(2M-H)^-$. The two substrates in Figure 4 provided ESI-negative at *m/z* 166 $(M-H)^-$, 122 (M-45) (4-nitrobenzoic acid), and *m/z* at 211 $(M-H)^-$, 167 $(M-45)^-$ and 423 $(2M-1)^-$ (2,4-dinitrobenzoic acid).

In agreement with previous spectrophotometric results (Table 2), no reduction was observed by HPLC(DAD)-MS of 2,4-dinitrophenol, 2-nitrobenzoic or 3-nitrobenzoic acid, suggesting that PnbA reductase was selective for some specific nitrocompounds as electron acceptors and with regard to position of the nitro substituent in the benzenic ring. The reduction of nitro in *para* position respective of the carboxylic group was required since 2- or 3-nitrobenzoic acid were not reduced by *L. plantarum* PnbA reductase. As mentioned above 4-

hydroxylaminebenzoic acid (100 μM) was not reduced to the corresponding amine by this reductase in presence of NADPH showing that the hydroxilamine was a final product of the catalytic reaction.

Enzyme reduction kinetics of PnbA reductase from L. plantarum. The reduction of 4-nitrobenzoic acid in presence of NADPH to give the corresponding hydroxylamine was rapid and linear in the first 10 min of incubation. Then, 3 min were subsequently used for kinetic studies by HPLC-DAD in order to calculate reaction rates and kinetic parameters. Figure 5 shows the Michaelis-Menten curves with the experimental reaction rates as a function of the concentration of substrate both for 4-nitrobenzoic acid and 2,4-dinitrobenzoic acid that gave 4-hydroxylaminobenzoic acid and 4-hidroxylamino-2-nitrobenzoic acid, respectively. Table 3 gives the kinetic values of $V_{max,}$ K_m and the ratio V_{max}/K_m. The calculated V_{max} was 2.3 times higher for reduction of 2,4-dinitrobenzoic acid than for 4-nitrobenzoic acid, whereas the K_m was much lower for 2,4-dinitrobenzoic acid (32-fold) than for 4-nitrobenzoate. This suggests a much higher affinity of *L. plantarum* reductase for 2,4-dinitrobenzoate than for 4-nitrobenzoate. Thus, V_{max}/K_m ratio was much higher for 2,4-dinitrobenzoate, indicating a higher suitability of this specific nitrocompound for reduction by PnbA reductase. On the other hand, the reaction rate to give the *p*-hydroxylamine derivative from 4-nitrobenzoic acid was similar in aerobic and anaerobic conditions (not shown), suggesting that this reductase was not highly affected by the oxygen as expected for an oxygen-insensitive nitroreductase. By following the elimination of NADPH (absorbance) and the corresponding formation of hydroxylamine by HPLC, the

452 stochiometry calculated was about 2 moles of NADPH consumed per mole of
453 hydroxylamine.
454
455 **DISCUSSION**
456 *L. plantarum* is a lactic acid bacteria species encountered in a wide range
457 of environmental niches and fermented foods, and with ability to survive gastric
458 transit and colonize the intestinal tract of human and other mammals (11, 12). In
459 this research, the gen (lp_0050) encoding a functional nitrobenzoate reductase
460 (Pnb A) from *L. plantarum* WCFS1 was cloned and expressed. This gen had
461 been previously annotated as a PnbA reductase during its complete DNA
462 sequencing (24). In addition, similar proteins annotated as PnbA reductases
463 were found in the complete genomes of several lactic acid bacteria belonging to
464 the *Lactobacillus* genera (*L. sakei*, *L. gasseri*, *L. reuteri*, *L. rhamnosus*, *L.*
465 *jhonsonii* and *L. delbrueckii*) as well as *Lactococcus* (*L. lactis*) and *Pediococcus*
466 (*P. pensosaceus*) genera (11, 35). However, none of these putative reductases
467 had been previously cloned and/or biochemically characterized. The amino acid
468 sequence of the PnbA reductase from *L. plantarum* showed a 23 % identity to
469 known nitroreductases from *P. putida*, and *R. euthropha,* but less than 11 % to
470 nitroreductases from enterobacteria such as *E. cloacae*, *E. coli*, and *S.*
471 *typhymurium*. A recombinant plasmid containing the *pnbA gen* was cloned into
472 *E*. *coli* and the encoded protein showed to be a catalytically active *L. plantarum*
473 PnbA reductase, resembling kinetic properties and inhibitor susceptibility of
474 flavin reductases. This enzyme was a FMN-flavoprotein (yellow colour) that
475 used both NADPH and NADH as electron donors, and it was inhibited by
476 dicoumarol and DPI. It could use a broad spectrum of electron acceptors such

as DCPIP, NBT, ferricyanide and quinones suggesting that its active site could accommodate molecules of varying size and structure as substrates. In contrast, it was more selective regarding the reduction of nitrocompounds, with only 4-nitrobenzoates being reduced which suggests a higher selectivity for two electron reduction of nitroaromatic groups. Moreover, *L. plantarum* PnbA reductase was not able to reduce potentially toxic pyridinium cations such as the herbicide paraquat and the naturally-occurring 2-methyl-β-carbolinium alkaloids. These compounds might generate free radicals and superoxide anion if reduced contributing to their toxicity (15-17, 31).

As occur with other characterized nitroreductases, the natural substrates of *L. plantarum* nitroreductase and its physiological and/or metabolic role in lactic bacteria are unknown (47). Since NAD(P)H is used as an electron donor, it has been speculated that nitroreductases could link pyrimidine nucleotide oxidation to the reduction of substrates to maintain the steady supply of oxidized pyrimidine nucleotides. *L. plantarum* PnbA reductase has the ability to utilize a variety of electron acceptors such as quinones and other substrates; thus, it could remove oxidants which otherwise would be reduced to a free-radical state and form intermediate species reacting with DNA and proteins. This raises the possibility that nitroreductases could somehow protect cells against oxidative stress and the toxic effects caused by exposure to quinones and/or related cellular oxidants (21, 32, 46). On the other hand, nitroreductases could function as specific enzymes involved in degradation pathways of nitrocompounds (47). In this regard, biodegradation of these compounds by using selective nitroreductases and microorganisms are of great interest for removal of environmental pollutants (45, 47). On the other hand, these

502 nitroreductases might be also of interest in the therapeutic action of
503 antimicrobial and anticancer prodrugs (4, 13, 55).

504 Living organisms can utilize nitroaromatic compounds which are
505 metabolized by nitroreduction catalyzed by two types of nitroreductases:
506 oxygen-sensitive and oxygen-insensitive (25, 57). Oxygen-sensitive such as
507 cytochrome P-450 oxidoreductase may catalyze the one-electron reduction of
508 nitro to the anion free radical which is reoxidized to the parent compounds by O_2
509 in a futile redox cycle producing superoxide anion. Oxygen-insensitve such as
510 NAD(P)H-quinone oxidoreductase and nitroreductases of enteric bacteria (*E.*
511 *coli*, *E. cloacae, Salmonella*) catalyze the two-electron reduction of the nitro
512 group to nitroso, hydroxylamine and/or amine derivatives (26, 39). The
513 functional PnbA from *L. plantarum* belongs to this latter family of
514 nitroreductases and reduced *p*-nitrobenzoates (4-nitrobenzoates and 2,4-
515 dinitrobenzoate) initially to 4-nitroso compounds which were rapidly converted
516 to hydroxylamino derivatives as final products (Figure 6). The enzyme was not
517 able to reduce the hydroxylamine further to *p*-aminobenzoate. This behaviour
518 agrees well with other microbial nitroreductases (39) that afford nitrosoaromatic
519 as a limiting step that quickly react to give hydroxylamine products (29, 49).

520 Nitroheterocyclic compounds constitute a wide range of chemicals whose
521 potent biological activity has significant human health and environmental
522 implications. Many nitrocompounds are well-known as potent mutagens and
523 carcinogens (1, 37, 38). The biological activity of these compounds is linked to
524 their reductive metabolism catalyzed by nitroreductases to hydroxylamine,
525 nitroso and electrophilic species (30, 34, 51). Bacterial nitroreductases such as
526 that one of *L. plantarum* perform two-electron reduction of nitroaromatic

527 compounds to hydroxylamine derivatives (6, 19, 36, 56). They are distributed
528 among different microorganisms suggesting a certain relevance within microbial
529 metabolic activities (11, 47). Nevertheless, certain aspects of their catalytic
530 process such as substrate specificity and reduction mechanism are not well
531 understood. Thus, nitroreductases from *E. cloacae* (6), *S. typhymurium* (53), *E.*
532 *coli* (22, 46, 58), *Ralstonia euthropha* (49), *Rhodobacter capsulatus* (40, 41),
533 *Pseudomonas* (7, 19, 27) are able to reduce a wide range of nitroaromatics
534 including nitroalquilbenzenes, nitrophenols, nitrobenzoates, nitrofurazone, and
535 *p*-nitroacetophenone, among others. Then, they appear to be relaxed on
536 substrate specificity and can reduce nitrocompounds and other electron
537 acceptors such as quinones (23, 36, 46). In this regard, the PnbA reductase
538 from *L. plantarum* was selective for reduction of nitrocompounds, and only
539 reduced those with a 4-nitrobenzoate moiety such as 4-nitrobenzoate and 2,4-
540 dinitrobenzoate. The efficiency (V_{max}/K_m) for reduction of 2,4-dinitrobenzoate
541 was much higher than for 4-nitrobenzoate, whereas it was unable to reduce
542 other nitrobenzoates or nitrophenols. This might suggest that this PnbA
543 reductase, and perhaps other nitroreductases annotated in lactic acid bacteria
544 (11, 35), may conform a class of more selective 4-nitrobenzoate
545 nitroreductases. Moreover, this reductase might have similar functional
546 properties in different lactic acid bacteria as it shows a high-scoring similarity
547 (32-43% identity) to other putative *p*-nitrobenzoate reductases from lactic
548 bacteria.

549 In conclusion, we have cloned, expressed and characterized a novel *p*-
550 nitrobenzoate (PnbA) reductase from *L. plantarum* WCFS1 that was previously
551 annotated on its complete genome sequence. Similar proteins seem to be

552 present in several other lactic acid bacteria. The PnbA reductase of *L.*
553 *plantarum* utilized a wide range of electron acceptors including quinones,
554 DCPIP, NBT and ferricyanide but it was not able to reduce pyridinium cations
555 such as paraquat and 2-methyl-β-carbolinium substances. However, it was
556 selective for nitro compounds reducing only 4-nitrobenzoates (4-nitrobenzoate
557 and 2,4-dinitrobenzoate) among a number of nitrocompounds assayed. This
558 PnbA nitroreductase may be distinct to other previously described bacterial
559 nitroreductases because of its selectivity for 4-nitrobenzoates. This different
560 selectivity could be due to that *L. plantarum* PnbA reductase exitibits a 23 %
561 identity to nitroreductases from *Psudomonas putida* and *R. euthropha* and even
562 was less identical (< 11 %) to enterobacteria PnbA (*e.g. E. cloacae*, *E. coli* and
563 *S. thyphymurium*. This report is the first characterization of a nitroreductase in
564 lactic acid bacteria that widely occur in foods, the environment and human
565 intestinal tract, and thereby these results might be of further interest.
566
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572
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795 Table 1. Reduction rates (μmol of substrate/min mg PnbA protein) of several
796 electron acceptors by PnbA reductase from *Lactobacillus plantarum* WCFS1.
797

Electron acceptor	*v* (μmol/min mg protein)[1]
DCPIP	6.6 ± 0.065
Cytochrome C	0.2 ± 0.03
Cytochrome C/menadione	1.51 ± 0.05
Ferricyanide	158 ± 1.4
NBT	1.9 ± 0.09

798
799 [1]*v* ± SD. Assays were carried out as indicated in experimental and performed at least in
800 triplicate.

Table 2. Activity of PnbA reductase from *L. plantarum* WCFS1 strain with different nitrocompounds, pyridinium cations and quinones as substrates.

Substrate	v (μmol NADPH/min mg prot)[1]
1,4-benzoquinone	12.3 ± 3.0
Menadione	2.26 ± 0.24
4-nitrobenzoic acid	2.92 ± 0.8
2,4-dinitrobenzoic acid	36.1 ± 6.1
2-nitrobenzoic acid	ND
3-nitrobenzoic acid	ND
2-hydroxy-3,5-dinitrobenzoic acid	ND
2,4-dinitrophenol	ND
2,4,6-trinitrophenol (picric acid)	ND
2-amino-4-nitrophenol	ND
Nitrobenzene	ND
5-nitroindazole	ND
4-nitropheny-β-D-glucopiranoside	ND
N-(2-nitrophenyl)proline	ND
N-(2-nitrophenyl)pipecolic acid	ND
N-(2,4-dinitrophenyl)glycine	ND
N-(2-nitrophenyl)glycine	ND
1,1-dimethyl-1,4-bypyridyl	ND
2-methyl-norharman	ND
2-methyl-harman	ND

[1]The enzymatic activity (*v* ± SD) was measured as indicated in Experimental section in presence of enzyme (1-5 μg/ml, 300 μM NADPH, and 100 μM substrate with the exception of menadione and 2,4-dinitrophenol that were assayed at 50 μM to avoid signal saturation. The rate was measured as the decrease of NAPDH (340 nm) in the first 1-2 min reaction at room temperature and the NADPH reduction in absence of substrate was substracted. ND, not detectable activity.

814 Table 3. Kinetic parameters (V_{max} and K_m) of the reduction of 4-nitrobenzoic
815 acid and 2,4-dinitrobenzoic acid by *L. plantarum* PnbA reductase.
816

Substrate	K_m (μM) ± SE	V_{max} ± SE (μmol/min mg protein)	V_{max}/K_m (l min^{-1} mg $prot^{-1}$)
4-nitrobenzoic acid	536 ± 102	15.1 ± 1.6	0.028
2,4-dinitrobenzoic acid	16.69 ± 6.4	35.5 ± 1.99	2.13

817 FIGURE CAPTIONS

818

819 Figure 1. Comparison of PnbA reductase from *L. plantarum* WCFS1 (LPL)
820 (accession CAD62742) and well-known PnbA reductase from *P. putida* (PPU)
821 (AAG01540), *R. euthropha* (REU) (YP_297809), and *E. cloacae* (ECO)
822 (Q01234). The ClustalW program was used to compare sequences. Conserved
823 residues of the nitroreductase family (Pfam 00881) are indicated (●). Residues
824 that are identical (*), conserved (:), or semiconserved (.) in all sequences of
825 the alignement are also indicated. Dashes represents gaps introduced to
826 maximize similarities.

827

828 Figure 2. SDS-PAGE analysis showing the PnbA expression and PnbA
829 purification from *L. plantarum* WCFS1 strain. Lanes: 1, soluble extract from *E.*
830 *Coli* JM109 (pURI3) cells; 2, extract from *E.coli* JM109 (pURI3-PnbA); 3, extract
831 from *E.coli* JM109 (pURI3-PnbA) not retained on the affinity His-Trap FF
832 column; 4, purified PnbA reductase protein retained on the affinity column and
833 eluted on 125 mM imidazole buffer. SDS-polyacrylamide gels were stained with
834 Coomassie blue. The position of some molecular mass markers (Bio-Rad) are
835 indicated on the letf.

836

837 Figure 3. Absorption spectra of the purified flavoprotein PnbA from *L. plantarum*
838 WCFS1 (a), and loss of the FMN bands following reduction with an excess of
839 sodium dithionite (b).

840

841 Figure 4. HPLC-MS (ESI) analysis of the reaction media of 4-nitrobenzoic acid
842 (280 nm) (a), and 2,4-dinitrobenzoic acid (243 nm) (b) reduction with *L.*
843 *plantarum* PnbA reductase. Electrospray spectra of in negative ion mode of the
844 corresponding hydroxylamine products are given. Reaction media in 100 mM
845 phosphate buffer pH 7.4, contained substrate (100 μM) NADPH (500 μM), PnbA
846 reductase (5 μg/ml) and were incubated at room temperature and reaction
847 stopped as indicated in Experimental.

848

849 Figure 5. Michaelis-Menten curves of the enzymatic reduction of 4-nitrobenzoic
850 acid (a) and 2,4-dinitrobenzoic acid (b) by PnbA reductase of *L. plantarum*.

Reaction media contained 4-nitrobenzoic acid or 2,4-dinitrobenzoic acid in 100 mM phosphate buffer pH 7.4, NADPH (500 μM), and PnbA reductase (0.25 μg/ml for 2,4-dinitrobenzoic acid and 2 μg/ml for 4-nitrobenzoic acid). Reaction was performed at room temperature for 3 min. Velocity (*v*) is given as μmol of corresponding hydroxylamine product RNHOH/min mg of PnbA reductase.

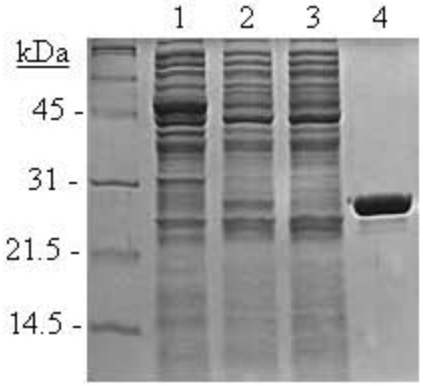
Figure 6. Selective reduction of 4-nitrobenzoic acid compounds to nitroso and hydroxylamino derivatives by PnbA reductase from the lactic acid bacterium *L. plantarum* WCFS1.

862
863

Figure 1


PPU MALLTDDFDAVVASRRAVRAFLP-TPISRKLISEIIDIARLAPSNSNTQPWSIHVLTGEP 59
REU -----MKVSQAVESRKSVRGFLP-NPIDPDTIRRVLAAAARAPSGGNLQPWHIHVVGGEA 54
LPL -----METIKAIHTRHSVRAFKD-DPIEPQLLTMIVTDAQQTPSWGNSQPWQVYIATGHA 54
ECL -----MDIISVALKRHSTKAFDASKKLTAEEAEKIKTLLQYSPSSTNSQPWHFIVASTE- 54
PPU KQALSALLGIAHNDPSADP---LAHLPDDLARKYRERQEKWGELFYGLHQIDKCDDAGRA 116
REU MDRLMDIMRQRVTEAPGGEEREYDIYPRELVSPYRDRRFEVGEALYRSLGIPREDKQRRL 114
LPL LTNIKQHYATAAEQGIAEDADLAKVHRGDFSAFASQNMGHWVGTFRPVIDSDP------T 108
ECL -EGKARVAKSAAGTYVFNERKMLDASHVVVFCAKTAMDDAWLERVVDQEEADGRFNTPEA 113
PPU RVSGLNFDFFGAPVGLIFTIDSNLKKYSWLDYGLFLQTLMLTARSRGLSTCPQVSFARFQ 176
REU AQFANNFAFFGAPLALFCSVDRRMGPPQWSDLGMYLQTVMLLLREEGLDSCAQECWAIYP 174
LPL TYWDSRANLYRAPAIAYLVLDANPNSWSIYDLGAFSQTLMLAATARGVQSVPSYELVKYP 168
ECL KAANHKGRTYFADMHRVDLKDDD--QWMAKQVYLNVGNFLLGVGAMGLDAVPIEGFD--A 169
PPU SLIKDFLHLDDS-QEIVCGMSLGYADENATVNSLRMPREMAQGFTHFMGFDR 227
REU QTIGGFLSLPPE-RMLFTGMAIGYEDPEAPANQLRAPRAPLEAFTEFMGI-- 223
LPL AIVRNALGMPAN-KVVAMGIALGYEKPEKPLNQFRTDRVATPQVLHIIN--- 216
ECL AILDEEFGLKEKGFTSLVVVPVGHHSVEDFNATLPKSRLPLSTIVTEC---- 217

Figure



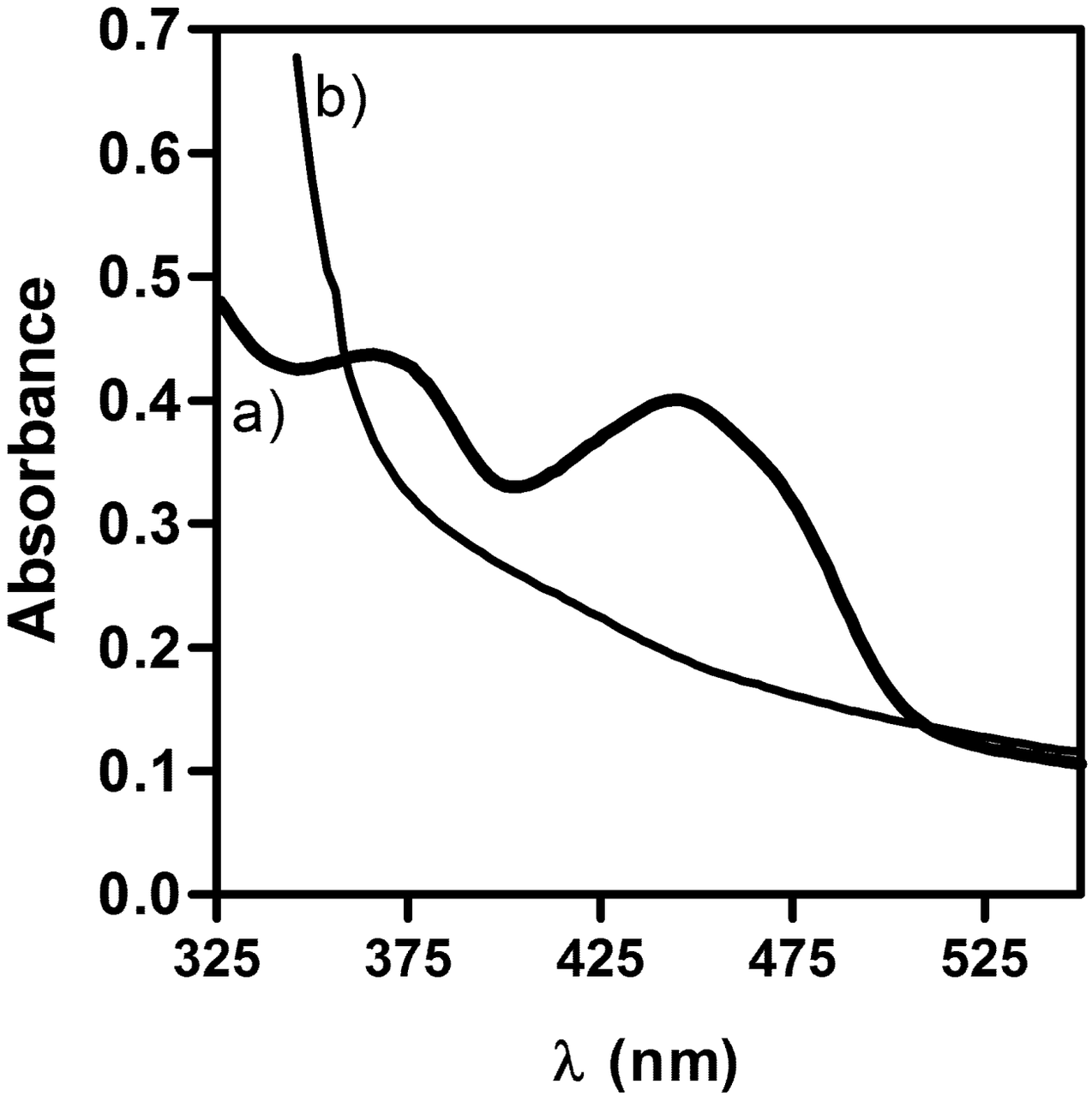
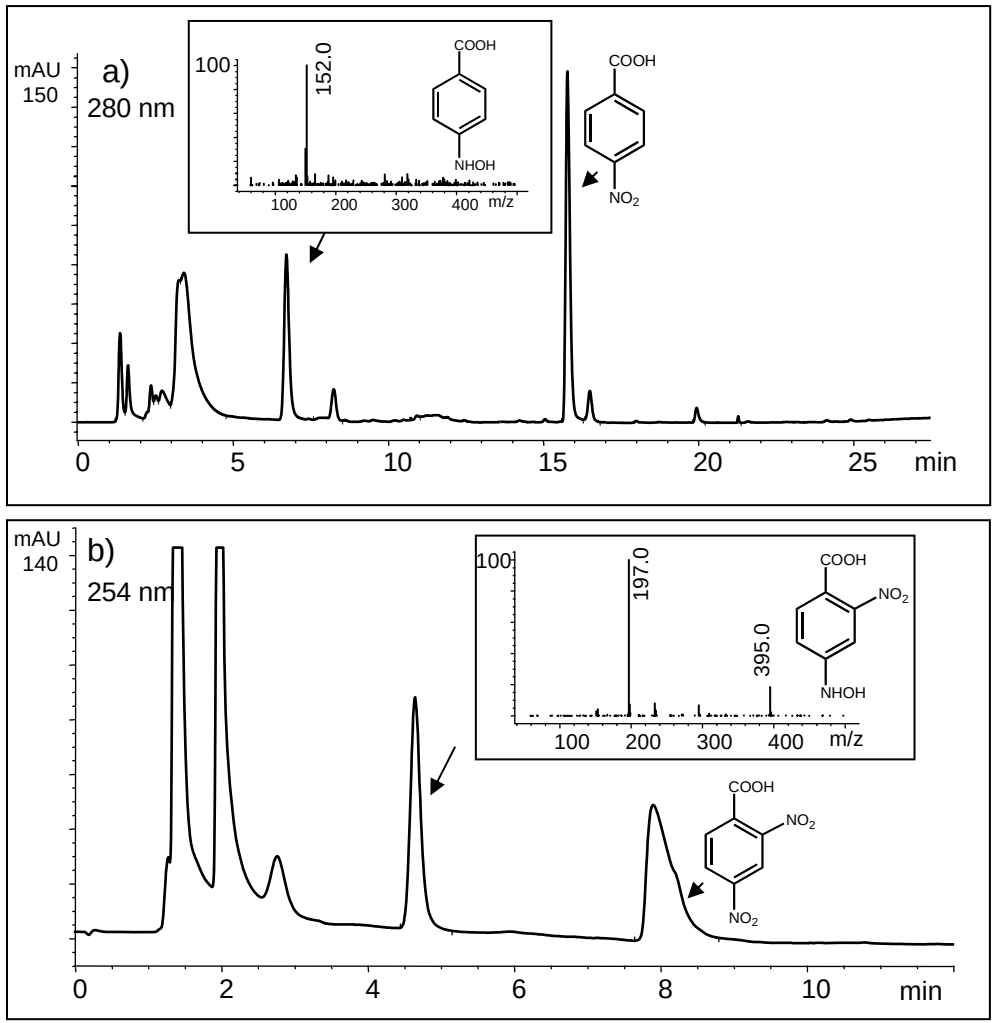


Figure 3


a)
280 nm
mAU
150
100
152.0
COOH
NHOH
100
200
300
400
m/z
COOH
NO2
0
5
10
15
20
25
min
b)
254 nm
mAU
140
100
197.0
395.0
COOH
NO2
NHOH
100
200
300
400
m/z
COOH
NO2
NO2
0
2
4
6
8
10
min

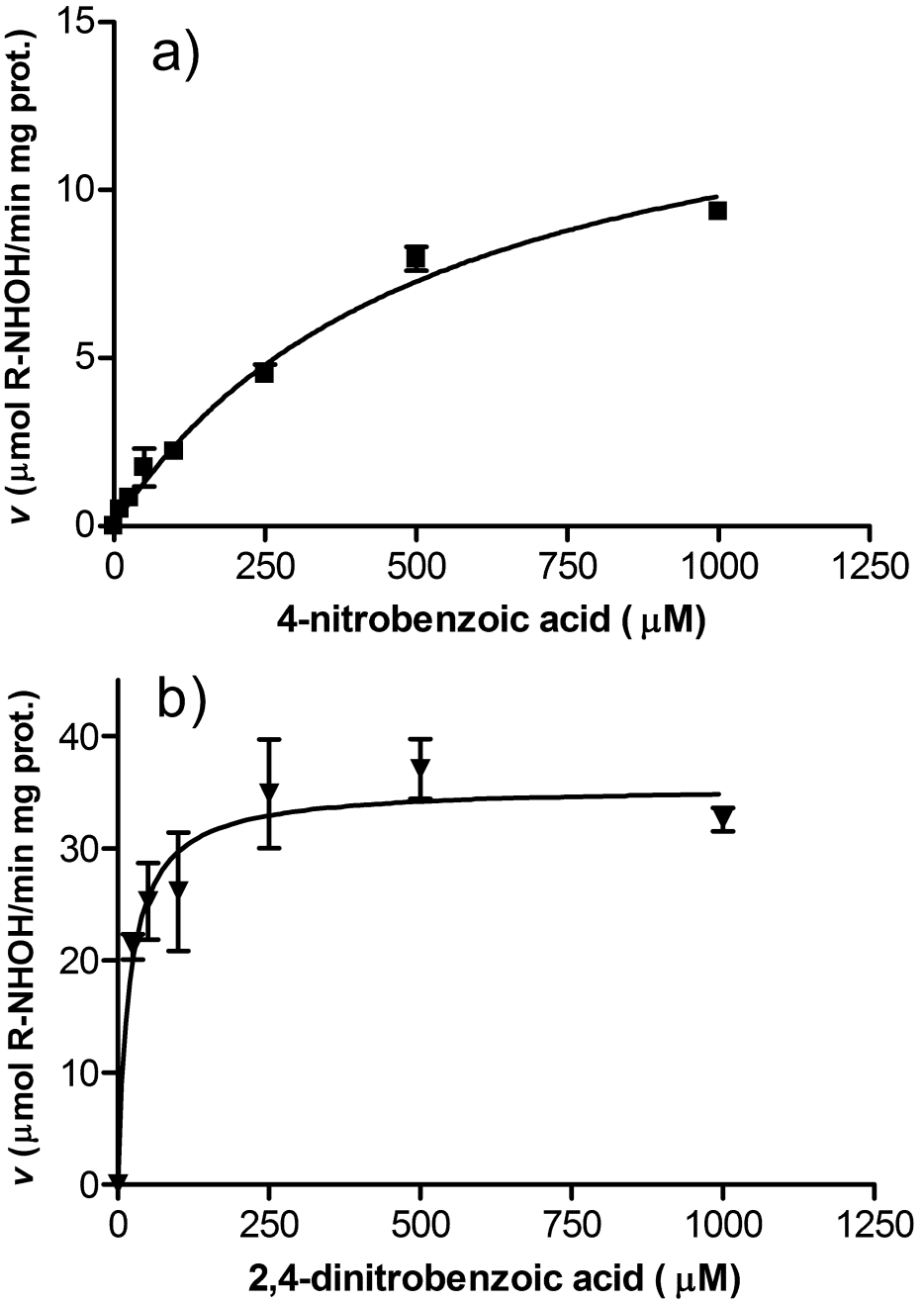
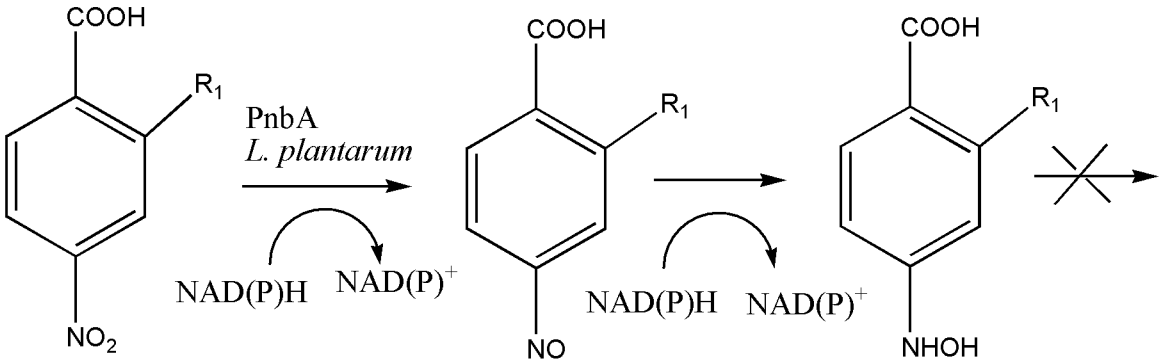

a)
v (μmol R-NHOH/min mg prot.)
15
10
5
0
0
250
500
750
1000
1250
4-nitrobenzoic acid (μM)
b)
v (μmol R-NHOH/min mg prot.)
40
30
20
10
0
0
250
500
750
1000
1250
2,4-dinitrobenzoic acid (μM)

Figure 5



R_1= H, NO_2

Figure 6