Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 854 (2007) 350–356
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Determination of oxalyl-coenzyme A decarboxylase activity in *Oxalobacter formigenes* and *Lactobacillus acidophilus* by capillary electrophoresis

Claudia Bendazzoli, Silvia Turroni, Roberto Gotti *, Stefano Olmo, Patrizia Brigidi, Vanni Cavrini

Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, Bologna 40126, Italy

Received 9 January 2007; accepted 20 April 2007
Available online 1 May 2007

Abstract

Oxalyl-coenzyme A decarboxylase (OXC) is a key enzyme in the catabolism of the highly toxic oxalate, catalysing the decarboxylation of oxalyl-coenzyme A (Ox-CoA) to formyl-coenzyme A (For-CoA). In the present study, a capillary electrophoretic (CE) method was proposed for the assessment of the activity of recombinant OXC from two bacteria, namely *Oxalobacter formigenes* DSM 4420 and *Lactobacillus acidophilus* LA 14. In particular, the degradation of the substrate Ox-CoA occurring in the enzymatic reaction could be monitored by the off-line CE method. A capillary permanently coated with polyethylenimine (PEI) was used and in the presence of a neutral background electrolyte (50 mM phosphate buffer at pH 7.0), a reversal of the electroosmotic flow was obtained. Under these conditions, the anodic migration of Ox-CoA (substrate) and For-CoA (reaction product) occurred and their separation was accomplished in less than 12 min. The CE method was validated for selectivity, linearity (range of Ox-CoA within 0.005–0.650 mM), sensitivity (LOD of 1.5 μM at the detection wavelength of 254 nm), precision and accuracy. Steady state kinetic constants (V_{max}, K_m or k') of OXC were finally estimated for both the bacteria showing that although *L. acidophilus* LA 14 provided a lower oxalate breakdown than *O. formigenes* DSM 4420, it could be a potentially useful probiotic in the prevention of diseases related to oxalate.

Keywords: Oxalyl-coenzyme A decarboxylase; *Oxalobacter formigenes*; *Lactobacillus acidophilus*; Capillary electrophoresis; Polyethylenimine; Kinetic parameters

1. Introduction

In humans, the absorption of oxalic acid by diets occurs mainly in the colon; the strong ability of oxalate as chelator of cations, calcium in principal, is the basis of oxalate accumulation which can result in a number of pathologic conditions such as: hyperoxaluria, urolithiasis, renal failure, etc. [1]. The oxalate-degrading activity of colonic anaerobic bacteria has been widely considered for the possible contribution in regulating the intestinal oxalate homeostasis [2]; in particular, the microorganism *Oxalobacter formigenes* found in the gastrointestinal tract of vertebrates, has been shown to depend totally on oxalate metabolism for energy and its role in scavenging dietary oxalate has been demonstrated [3–5]. In this microorganism, oxalyl-coenzyme A decarboxylase (OXC) is the key enzyme in the catabolism of the highly toxic oxalate, catalysing the decarboxylation of oxalyl-coenzyme A (Ox-CoA) to formyl-coenzyme A (For-CoA) [6]. The structures of Ox-CoA and For-CoA and the scheme of catabolism of oxalate by *O. formigenes* are reported in Fig. 1. Besides *O. formigenes*, several bacteria have been recognized in intestinal tract as oxalate-degraders [7]. Furthermore, bacteria industrially employed as probiotics have shown evidence for a potential oxalate-degrading activity [8] and they are now objects of study as potential food additives in the prevention of oxalate-related diseases. The OXC activity in bacteria is conventionally screened by spectrophotometric methods where the decarboxylation of the substrate is estimated by the formate amount yielded as the result of coupled reactions involving, eventually, the reduction of NAD to NADH [6]. The main drawback of these coupled methods is the low specificity of analysis due to the complexity of the enzymatic reaction mixture [9]. The required high selectivity for reliable and fast determinations

* Corresponding author. Tel.: +39 051 2099729; fax: +39 051 2099734.
E-mail address: roberto.gotti@unibo.it (R. Gotti).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.04.027

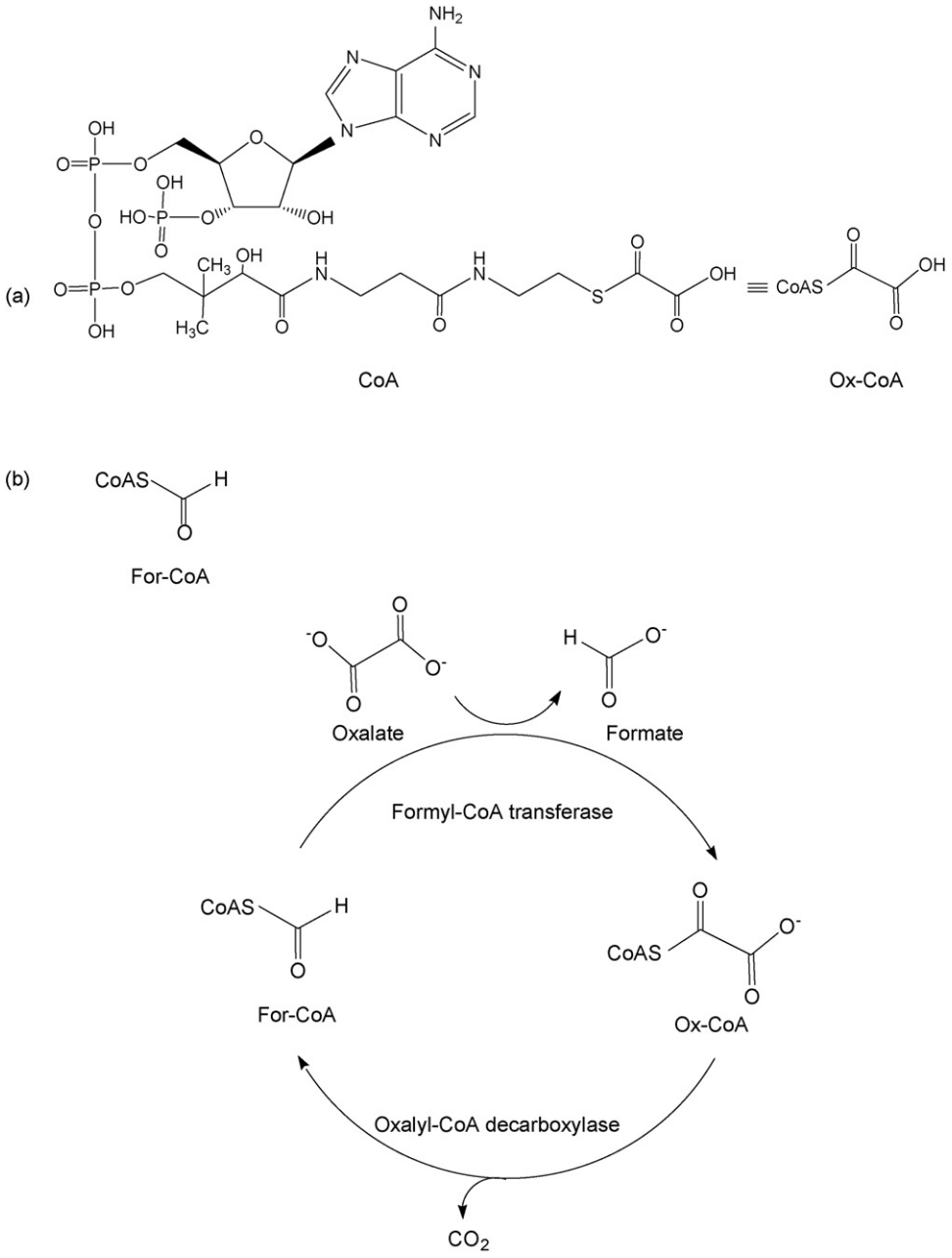

Fig. 1. Structures of oxalyl-CoA (a), formyl-CoA (b) and catabolic pathway of oxalate by *O. formigenes*.

of enzymatic activity can be fulfilled by applying suitable separation methods; among these, capillary electrophoresis (CE) has shown to be very attractive for the rapid analysis times, automation of the analytical procedure and small consumption of both samples and reagents. As recently reviewed by Glatz [10], CE can be exploited as an efficient separation technique for the quantitation of reaction product(s) or substrate(s) after the enzyme reaction is occurred (off-line assay). Besides these classic approaches, enzymatic reactions can be also advantageously performed in CE by separately introducing inside the capillary both the enzyme and substrate(s) and giving them the possibility to react with each other by applying the electrical field. By exploiting the mobility differences, the products of the enzymatic reaction obtained on-line, can be separated and thus quantified, in a same run (on-line assay).

In our previous paper, an off-line CE method was proposed in evaluating the OXC activity in *Bifidobacterium lactis*, a probiotic bacterium widely used in dairy fermented products and in pharmaceutical preparations [8]. Recent evidences of the efficacy of hyperoxaluria treatment with a lactic acid bacteria mixture containing the probiotic *Lactobacillus acidophilus* [11,12], suggested the determination of its oxalate-degrading activity. An "in vitro" screening of this activity was carried out on several *Lactobacillus* strains isolated from functional foods and pharmaceutical preparations. To this regard CE using a polyethylenimine (PEI) coated capillary [13], was applied for the

first time to the determination of Formyl-CoA transferase activity and oxalyl-CoA decarboxylase activity. The method provided a reversed electroosmotic flow (from cathode to anode) and allowed for a fast separation of the reaction substrate Ox-CoA and the product For-CoA [14]. In the present study, the previously proposed CE method has been implemented, validated and applied to the assessment of the activity of recombinant OXC from *L. acidophilus* LA 14 and a comparison with the activity of OXC from *O. formigenes* DMS 4420, was carried out. The kinetic parameters of oxalyl-CoA decarboxylase were finally estimated in both the studied bacteria.

2. Experimental

2.1. Materials

Polyethylenimine (PEI), phthalic acid and dimethyl sulfoxide (DMSO) (the marker of electroosmotic flow), were provided by Sigma–Aldrich (Milan, Italy). Hydrogen phosphate anhydrous sodium salt, sulfuric acid, sodium hydroxide and all the other chemicals of analytical grade, were purchased from Carlo Erba Reagenti (Milan, Italy). Water used for the preparation of solutions and running buffers, was purified by a Milli-RX apparatus (Millipore, Milford, MA, USA).

2.2. Standard substances

The standard substance Ox-CoA is not commercially available and it was synthesized by reaction of coenzyme A with thiocresoxalic acid following a literature procedure [15]. The final product, characterized by NMR, was titrated by a spectrophotometric assay (UV absorbance at 590 nm) using an enzymatic method (Oxalate Diagnostic, Prisma, Milan, Italy) based on the oxidation of oxalate, by oxalate oxidase [16]. Briefly, an accurately weighted amount of the synthesized Ox-CoA was dissolved in water and 50 μL aliquot of the obtained solution was neutralized to pH 7.0 by adding a 5 μL volume of sodium hydroxide (0.1 M). After stirring, the mixture was stored at 37 °C for 15 min in the presence of the enzymatic reagents from the Oxalate Diagnostic kit; finally the mixture was acidified to pH 5.0 by addition of hydrochloric acid (0.1 M). The concentration of synthesized Ox-CoA was determined by comparison with a reference standard solution of oxalate. The Ox-CoA solution was then diluted and stored at the final concentration of 2.0 mM at the temperature of −80 °C.

Formyl-CoA was synthesized by ester interchange between CoA and thiocresyl formate [17]; the identity of the obtained compound was confirmed by NMR spectrum. The synthesized For-CoA was not further purified nor titrated, because it was used as standard only for qualitative analysis.

2.3. Apparatus

Electrophoretic experiments were performed on a BioRad Biofocus 2000 instrument from BioRad (Hercules, CA, USA). The data were collected on a personal computer equipped with the devoted BioRad integration software. The separations were carried out on a PEI-coated capillary of 50 μm internal diameter (ID) with a total length of 36 cm (effective length 31.5 cm). The procedure for the coating of the capillary using PEI was based on a published method [13]. In order to obtain high repeatability of migration times, the capillary was rinsed between the runs with water (2 min) and separation buffer (2 min). The electrophoretic runs were performed at a constant voltage of 10 kV (anodic detection) with controlled temperature (25 °C). The samples were injected hydrodynamically using a pressure of 2 psi s (1 psi = 6894 Pa); the detection wavelength was 254 nm.

2.4. Solutions

Phosphate buffer used as the background electrolyte (BGE) was prepared at the concentration of 50 mM in water and the pH was adjusted at the desired value using a 0.1 M aqueous solution of phosphoric acid. Sulfuric acid aqueous solution was used at the concentration of 0.1N. The experiments for the evaluation of OXC activity were performed on solutions of the substrate (Ox-CoA) in a buffer constituted of phosphate (0.1 M; pH 6.8), thiamine pyrophosphate (60 μM) and $MgCl_2$ (6.0 mM).

2.5. Calibration curve

Linearity of the response was investigated for the substrate (Ox-CoA) in the concentration range of 0.005–0.650 mM in the buffer solution by using phthalic acid as internal standard (final concentration of 0.60 mM). Triplicate injections were made for each of the samples and the mean corrected peak area (area/migration time) ratios of the analyte to that of the internal standard were plotted against the concentrations to obtain the calibration graph by linear regression analysis.

2.6. Oxalyl-CoA decarboxylase reaction

The evaluation of OXC activity was carried out by measuring the consumption of the substrate Ox-CoA. The reaction mixture was constituted of the substrate Ox-CoA dissolved in the buffer (see Section 2.4). The reaction started by adding the soluble fraction (100 ng/μL protein) of a recombinant *Escherichia coli* clone overexpressing *L. acidophilus* LA 14 OXC enzyme. Likewise, the reaction was performed using the soluble fraction of a recombinant *E. coli* clone overexpressing *O. formigenes* DSM 4420 OXC enzyme (100 ng/μL protein).

Each mixture (total volume of 165 μL) was incubated at 37 °C for a period ranging from 5 to 90 min (*L. acidophilus* LA 14) or 30–120 s (*O. formigenes* 4420) and quenched with 16.5 μL of sulfuric acid (0.1N). Before CE analysis, the reaction mixtures were neutralized by adding 20 μL of NaOH (0.1 M) containing phthalic acid (0.60 mM) as the internal standard.

2.7. Kinetic analysis of oxalyl-CoA decarboxylase

The kinetic studies were performed using the cytoplasmatic extracts of recombinant *E. coli* clones overexpressing either *L. acidophilus* LA 14 oxalyl-CoA decarboxylase or *O. formigenes* DSM 4420 oxalyl-CoA decarboxylase. The evaluation of the

Ox-CoA degradation rate was carried out by analysing the different reaction mixtures containing different substrate Ox-CoA levels, ranging within 0.010–0.600 mM.

The data were fitted to the single-site Michaelis–Menten (hyperbolic substrate concentration dependence) and autoactivation (sigmoidal substrate concentration dependence) models. The autoactivation model is expressed by the Hill equation: $v = V_{max}[S]^h/(k' + [S]^h)$, where v is the rate of the metabolic reaction, $[S]$ the substrate concentration, V_{max} the maximum rate, k' is a constant of the autoactivation model that is equivalent to Michaelis–Menten K_m for $h = 1$, where h is the Hill coefficient. Standard parameters such as the determination coefficient (r^2) and standard errors of the parameter estimates were used to determine the quality of a fit to a specific model. Both fits and determination of the apparent enzyme kinetic parameters, K_m, V_{max}, k' and h, were calculated by non-linear regression analysis using GraphPad Prism version 4.0 (GraphPad software, San Diego, CA, USA).

3. Results and discussion

The evaluation of oxalyl-CoA decarboxylase and formyl-CoA transferase activity in oxalate-degrading bacteria can be conveniently determined by the enzymatic mixture variations in concentration of both Ox-CoA and For-CoA which perform as substrate/product in the oxalate degradation cycle (Fig. 1). A capillary electrophoretic approach has been previously applied in our laboratory to these evaluations in *L. acidophilus*; in particular the analysis of the anionic species Ox-CoA and For-CoA was carried out under co-electroosmotic flow mode [14]. In the present study, the proposed CE method has been implemented, validated and applied to estimate the kinetic parameters of OXC from *L. acidophilus* LA 14 and *O. formigenes* DSM 4420.

3.1. Method development

The analysis of anionic species under reversed EOF was found to be advantageous because of the shorter analysis time and improved peak shapes obtained in comparison to the conventional counter-electroosmotic migration [18,19]. In general, the inversion of the EOF is achieved by introducing cationic additives into the BGE to provide a dynamic coverage of the inner capillary silica surface that results positively charged. Recently, Righetti et al. summarized the different approaches in quenching and/or modulating the EOF by using different classes of compounds as BGE additives [20]. Most commonly, surfactants such as hexadecyltrimethylammonium bromide (CTAB) even if used at concentrations below the critical micelle concentration (cmc), reverse the EOF direction. In our preliminary experiments however, the addition of CTAB to the electrophoretic BGE resulted to be useless in analysis of Ox-CoA and For-CoA, likely due to their electrostatic as well as hydrophobic interactions with the surfactant monomers. Alternatively, the use of a capillary coated with a cationic layer, allowed the EOF to be reversed, avoiding the addition to the BGE of interfering additives. In this regard, PEI resulted to be a useful compound for the preparation of permanent coated capillaries: in fact as it has been previously proposed [13], PEI is a polycationic branched polymer able to be permanently adsorbed by the silica surface of the capillary wall. Detailed studies were reported on the EOF variations in PEI-coated capillaries using different running buffer nature and pH [13,21–24]. In the present study, a fused silica capillary was modified by adsorption of a 10% (w/v) PEI aqueous solution and it was used, in the presence of a BGE consisting of phosphate running buffer (50 mM, pH 7.0), in the analysis of actual enzymatic mixtures containing Ox-CoA and For-CoA. By applying a constant voltage of 10 kV (anodic detection), the obtained current was about 35 μA and the electroosmotic mobility, measured using DMSO (0.01% aqueous solution) as the neutral marker, was determined to be -6.0×10^{-5} cm^2 s^{-1} V^{-1}. Under these conditions the complete separation of Ox-CoA and For-CoA was accomplished in less than 12 min (Fig. 2).

3.2. Method validation

3.2.1. Linearity and sensitivity

Calibration curve was obtained by plotting the corrected peak area ratios of Ox-CoA to the internal standard (phthalic acid) (Y), versus the corresponding concentrations of the analyte (C; mM). By linear regression analysis the following equation was obtained: $Y = 40.962\ (\pm 0.381)\ C + 0.291\ (\pm 0.117)$; $r^2 = 0.999$.

The sensitivity data at the detection wavelength of 254 nm estimated as LOD (S/N = 3) and LOQ (S/N = 10) were found to be 1.5 and 5.0 μM (RSD = 3.2%; $n = 3$), respectively.

3.2.2. Selectivity and reproducibility

Identification of the studied analytes was performed by comparison of the migration times obtained in actual samples with those of the standard solutions. Furthermore, spiking experiments (standard addition method) were performed to confirm the peak identity.

The repeatability of the separation system was evaluated by replicated analysis of solution (0.150 mM) of Ox-CoA and For-CoA; the relative standard deviations of migration time were 1.07% and 1.05% ($n = 5$, intra-day) and 3.56% and 3.72% ($n = 15$, inter-day), for Ox-CoA and For-CoA, respectively. The RSD% of the corrected peak area ratio (analytes to internal standard) were 1.02% and 0.98% ($n = 5$, intra-day) for Ox-CoA and For-CoA, respectively, and it was found to be less than 6.0% over a three consecutive days experiments ($n = 15$).

3.2.3. Recovery studies

The accuracy of the method was evaluated by comparing the quantitative results obtained by the analysis of actual samples (enzymatic reaction mixtures) using the proposed CE method with those obtained by applying a standard spectrophotometric method based on the coupled assay described in experimental section (see Section 2.2). Precisely, aliquots of the stock solution of the substrate Ox-CoA corresponding to about 0.300 mM were mixed with the reaction cofactors and added, in the order with sulfuric acid solution (0.1N) and OXC from *O. formigenes* DSM 4420. Under these conditions, the pH requirement for the enzymatic reaction was not fulfilled and the oxalate degradation activity did not occur. Afterward, the samples were subjected

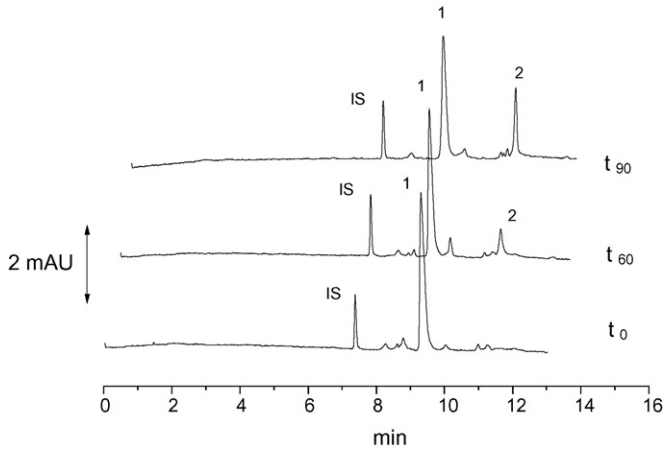

Fig. 2. Electropherograms of enzymatic reaction mixtures in presence of OXC from *L. acidophilus* LA 14 after different incubation times (0, 60 and 90 min); the initial concentration of Ox-CoA was 0.150 mM. *Conditions*: PEI-coated capillary (effective length 31.5 cm; 50 μm ID); the BGE is a 50 mM phosphate buffer (pH 7.0); applied voltage −10 kV; wavelength at 254 nm; hydrodynamic injection at 2 psi s; capillary temperature at 25 °C. *Symbols*: IS, internal standard; (1) Ox-CoA; (2) For-CoA.

to analysis of Ox-CoA using both the techniques (CE and spectrophotometric coupled assay); the obtained results (mean of five independent samples) were 0.301 mM (RSD 0.49%) and 0.307 mM (RSD 0.20%), respectively. The variance ratio *F*-test values calculated at 95% confidence level did not exceed the tabulated value, thus indicating no significant difference between the two applied methods.

3.3. Application to kinetic monitoring of OXC from O. formigenes DSM 4420 and L. acidophilus LA 14

The time course of substrate degradation by OXC from both of the bacteria was determined by analysis of Ox-CoA carried out at different times of the enzymatic reactions; electropherograms of the analysed actual enzymatic mixtures using *L. acidophilus* LA 14 at time zero and after 60 and 90 min are reported in Fig. 2. The corrected peak area ratios (remaining substrate versus internal standard) were plotted against the reaction time to obtain the degradation kinetics for both the bacteria (Fig. 3a and b). The concentration of Ox-CoA in these experiments was 0.150 mM. As expected, owing to the demonstrated dependence on oxalate metabolism for energy, the profiles of degradation of Ox-CoA showed that *O. formigenes* DSM 4420 OXC activity is significantly higher than that observed in *L. acidophilus* LA 14. However, although the latter showed to be not specifically involved in oxalate metabolism, the obtained results confirmed its role as oxalate-degrading microorganism. For a more in depth characterization of OXC activity, kinetic parameters were also estimated.

3.3.1. Kinetic parameters of OXC from O. formigenes DSM 4420

The time course evaluated in *O. formigenes* DSM 4420 (Fig. 3a), suggested that the degradation of the substrate Ox-CoA

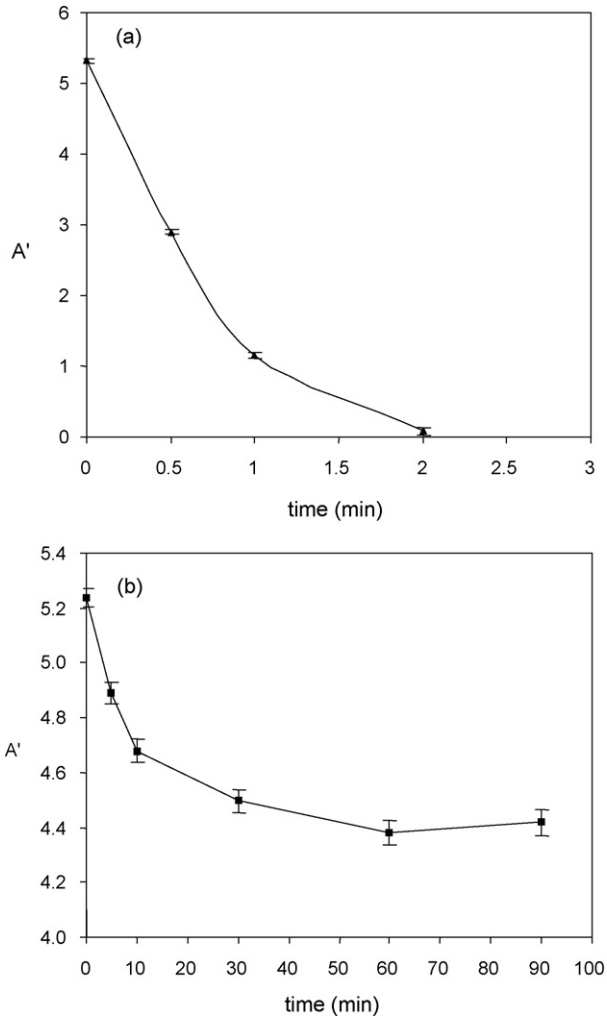

Fig. 3. Degradation kinetics of Ox-CoA by OXC from *O. formigenes* DSM 4420 (a) and from *L. acidophilus* LA 14 (b) as estimated by CE method. The concentration of Ox-CoA was 0.150 mM. A' is the corrected peak area ratio.

completely occurred within 2 min, thus the initial rate (v_0) was estimated by quenching the enzymatic reaction after 5 s. Under this condition, the observed decrease in the peak area of the substrate was corresponding to about 5%. Five different Ox-CoA levels within the concentration range 0.010–0.310 mM, were reacted with the enzyme OXC in triplicate; after 5 s the reactions were quenched and the remaining Ox-CoA amount (expressed as μmol) for each of the levels, was determined by the validated CE method using external standardization. The obtained data (mean of three measurements) were used to evaluate the initial rate of the metabolic reaction at different substrate concentrations. The kinetic parameters of OXC in the considered substrate concentration range were obtained by the Michaelis–Menten plot as shown in Fig. 4. The curve fitting to $v = V_{max}[S]/(K_m + [S])$ gave a V_{max} of 8.47 ± 0.51 μmol/min/mg and a K_m of 34.3 ± 6.6 μM, with a r^2 value of 0.98. The obtained values were basically in agreement with those previously reported [9].

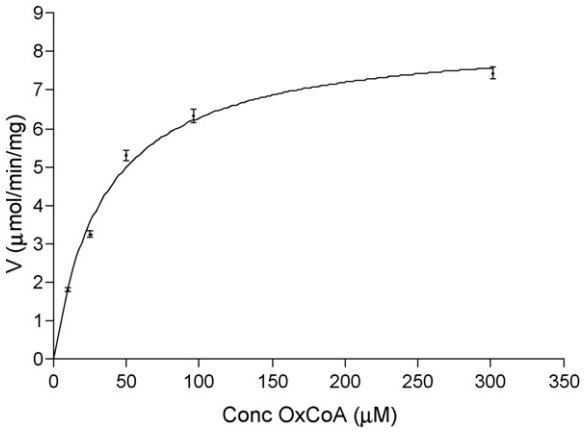

Fig. 4. Ox-CoA degradation rate vs. Ox-CoA concentration for OXC from *O. formigenes* DSM 4420 fitted with the Michaelis–Menten model.

3.3.2. Kinetic parameters of OXC from L. acidophilus LA 14

The kinetic parameters were estimated in *L. acidophilus* LA 14 by following the same experimental approach as for *O. formigenes* DSM 4420; however, since *L. acidophilus* LA 14 had shown a slower time course, a ∼5% degradation of the initial substrate amount was achieved after 5 min of incubation. Seven different Ox-CoA levels within the concentration range of 0.037–0.574 mM were reacted with the enzyme in triplicate and the remaining Ox-CoA amount (μmol) for each of the levels, was determined by the CE method.

The kinetic parameters of OXC in the studied substrate concentration range were obtained by curve fitting to $v = V_{max}[S]^h/(k' + [S]^h)$ as shown in the graph of Fig. 5. Since the equation fitted with a sigmoidal curve, a positive cooperativity between enzyme and substrate was hypothesized. The best fitting of the curve was found to be at h value of 4, where h is the number of cooperative binding sites. Under these fitting conditions, a V_{max} of 0.0383 ± 0.00134 μmol/min/mg and a k' of 82.6 ± 4.2 μM were found. The good determination coefficient (r^2 value of 0.97) and the obtained high repeatability of the system suggested that, even using a relatively low number of experimental points in the low concentration region, the obtained enzyme kinetic parameters can be fairly accepted.

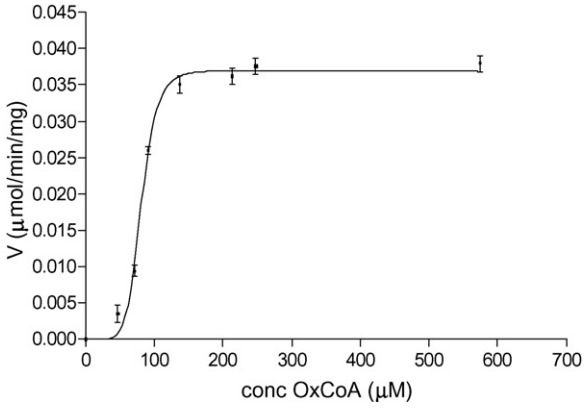

Fig. 5. Ox-CoA degradation rate vs. Ox-CoA concentration for OXC from *L. acidophilus* LA 14 fitted with the Hill equation model.

The pronounced differences in the kinetic parameter values reported for the oxalyl-CoA decarboxylase activity from the two studied bacteria, confirmed the lower oxalate-degrading activity in *L. acidophilus*, in agreement with its known ability to use substrate other than oxalate for growth. Furthermore, the supposed positive cooperativity and the putative presence of four interacting binding sites suggested a multimeric (presumably tetrameric) nature of *Lactobacillus* OXC. It is noteworthy that the crystal structure of *O. formigenes* OXC showed a tetrameric arrangement [9].

4. Conclusion

A previously developed CE method has been implemented and validated for the quantitation of Ox-CoA in a wide range of concentration in order to be applied in evaluating the consumption of the toxic compound oxalate by the oxalate-degrading bacteria *O. formigenes* DSM 4420 and *L. acidophilus* LA 14. The method has allowed the determination of kinetic parameters of OXC from both the bacteria, showing that although *Lactobacillus* provided a lower oxalate breakdown than *Oxalobacter*, the first could be a potentially useful probiotic in the prevention of diseases related to oxalate. The proposed CE method showed to be reliable and easy to apply thus representing a suitable and convenient alternative to the spectrophotometric method of analysis suffering for poor selectivity. The kinetic data obtained for *O. formigenes* DSM 4420, were found to be in good agreement with those reported in the literature and obtained by applying a gradient elution HPLC method.

Acknowledgement

This work has been supported by a grant from MIUR (PRIN 2004).

References

[1] H.E. Williams, L.H. Smith, Am. J. Med. 45 (1968) 715.
[2] M.J. Allison, C.M. Hook, D.B. Milne, S. Gallagher, R.V. Clayman, J. Nutr. 116 (1986) 455.
[3] K.A. Dawson, M.J. Allison, P.A. Hartman, Appl. Environ. Microbiol. 40 (1980) 833.
[4] H. Sidhu, M.E. Schmidt, J.G. Cornelius, M.E. Thamilselvan, S.R. Khan, A. Hesse, A.B. Peck, J. Am. Soc. Nephrol. 10 (1999) S334.
[5] M. Hatch, J. Cornelius, M. Allison, H. Sidhu, A. Peck, R.W. Freel, Kidney Int. 69 (2006) 691.
[6] A.L. Baetz, M.J. Allison, J. Bacteriol. 171 (1989) 2605.
[7] S. Hokama, C. Toma, M. Iwanaga, M. Morozumi, K. Sugaya, Y. Ogawa, Int. J. Urol. 12 (2005) 533.
[8] F. Federici, B. Vitali, R. Gotti, M.R. Pasca, S. Gobbi, A.B. Peck, P. Brigidi, Appl. Environ. Microbiol. 70 (2004) 5066.
[9] C.L. Berthold, P. Moussatche, N.G.J. Richards, Y. Lindqvist, J. Biol. Chem. 280 (2005) 41645.
[10] Z. Glatz, J. Chromatogr. B 841 (2006) 23.
[11] C. Campieri, M. Campieri, V. Bertuzzi, E. Swennen, D. Matteuzzi, S. Stefoni, F. Pirovano, C. Centi, S. Ulisse, G. Famularo, C. De Simone, Kidney Int. 60 (2001) 1097.
[12] J.C. Lieske, D.S. Goldfarb, C. De Simone, C. Reigner, Kidney Int. 68 (2005) 1244.
[13] F.B. Erim, A. Cifuentes, H. Poppe, J.C. Kraak, J. Chromatogr. A 708 (1995) 356.

[14] S. Turroni, B. Vitali, C. Bendazzoli, M. Candela, R. Gotti, F. Federici, F. Pirovano, P. Brigidi, J. Appl. Microbiol., doi:10.1111/j.1365-2672.2007.03388.x.
[15] J.R. Quayle, Biochim. Biophys. Acta 57 (1962) 398.
[16] M.F. Laker, A.F. Hofmann, B.J. Meeuse, Clin. Chem. 26 (1980) 827.
[17] P.C. Bax, W. Stevens, Recl. Trav. Chim. Pays-Bas Belg. 89 (1970) 265.
[18] A.J. Zemann, J. Chromatogr. A 787 (1997) 243.
[19] S.M. Masselter, A.J. Zemann, J. Chromatogr. A 693 (1995) 359.
[20] P.G. Righetti, C. Gelfi, R. Sebastiano, A. Citterio, J. Chromatogr. A 1053 (2004) 15.
[21] F.B. Erim, J. Chromatogr. A 768 (1997) 161.
[22] A. Cifuentes, H. Poppe, J.C. Kraak, F.B. Erim, J. Chromatogr. B 681 (1996) 21.
[23] E. Córdova, J. Gao, G.M. Whitesides, Anal. Chem. 69 (1997) 1370.
[24] M. Spanilá, J. Pazourek, J. Havel, J. Sep. Sci. 29 (2006) 2234.