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Research Article

Pseudomonas aeruginosa amidase: Aggregation in recombinant *Escherichia coli*

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The effect of cultivation parameters such as temperature incubation, IPTG induction and ethanol shock on the production of *Pseudomonas aeruginosa* amidase (E.C.3.5.1.4) in a recombinant *Escherichia coli* strain in LB ampicillin culture medium was investigated. The highest yield of soluble amidase, relatively to other proteins, was obtained in the condition at 37°C using 0.40 mM IPTG to induce growth, with ethanol. Our results demonstrate the formation of insoluble aggregates containing amidase, which was biologically active, in all tested growth conditions. Addition of ethanol at 25°C in the culture medium improved amidase yield, which quantitatively aggregated in a biologically active form and exhibited in all conditions an increased specific activity relatively to the soluble form of the enzyme. Non-denaturing solubilization of the aggregated amidase was successfully achieved using L-arginine. The aggregates obtained from conditions at 37°C by Furier transform infrared spectroscopy (FTIR) analysis demonstrated a lower content of intermolecular interactions, which facilitated the solubilization step applying non-denaturing conditions. The higher interactions exhibited in aggregates obtained at suboptimal conditions compromised the solubilization yield. This work provides an approach for the characterization and solubilization of novel reported biologically active aggregates of this amidase.

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1 Introduction

Microbial amidases of the thiol nitrilases family have numerous potential applications. Bacterial amidases (acylamide amidohydrolases, EC 3.5.1.4) catalyze the hydrolysis of amides into the corresponding carboxylic acids and ammonia [1–3]. The importance of these hydrolases in biotechnology is rapidly growing, as the potential applications span through chemical, pharmaceutical industries and in bioremediation of toxic amides [4, 5]. Additionally, these amidases display acyl transferase activity in the presence of hydroxylamine [6], the resulting hydroxamic acid derivatives are known for chelating properties [7, 8]. Some of which are described as potent inhibitors of metalloproteases and have been investigated in therapeutic applications [9, 10], and others can be used in wastewater treatments in the removal of metal ions [11].

This work reports the optimized production of *Pseudomonas aeruginosa* wild-type amidase (E.C. 3.5.1.4.) overproduced in a recombinant *Escherichia coli* strain pKK WT. The bacteria *E. coli* is often the choice of host in recombinant DNA technology, however with the disadvantage of aggregates formation when protein is overexpressed [12]. High levels of expression of recombinant proteins often result in aggregation and accumulation preventing the production of soluble protein at high yields [13]. Such dense aggregates of misfolded polypeptide, often referred as inclusion bodies,

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Abbreviations: FTIR, Fourier transform infrared spectroscopy; **GdnHCl,** guanidine hydrochloride; **IPTG,** isopropyl-β-D-thiogalactoside; **LB,** Luria Bertani

are formed intracellularly [14–16]. Although the formation of these insoluble aggregates could facilitate protein purification, the refolding into biological active forms often is a complicated procedure resulting in poor recovery [17, 18]. Therefore, most efforts have been addressed for the reduction of aggregates formation, with production of recombinant proteins in a soluble form in vivo and subsequently purify using conventional techniques.

Remarkably, some reports have identified enzymatic activity associated to aggregates of recombinant enzymes [19–21]. For such cases, aggregates mild solubilization has demonstrated a high recovery of bioactive protein when compared with the classical protocol using chaotropic agents [22–25]. However, the occurrence of functional aggregates is still poorly understood. Additional investigation over aggregated protein conformation is needed, since it can be variable depending on the protein and cell physiological conditions [26]. Spectroscopic Fourier transform infrared spectroscopy (FTIR) analysis as proved to be an important tool in this analysis [15, 18, 27, 28].

Our work addresses the effect and combination of parameters, such as cultivation temperature of Luria Bertani (LB) ampicillin supplemented medium, isopropyl-β-D-thiogalactoside (IPTG) concentration and ethanol shock, in the aggregation of amidase in recombinant *E. coli*. Spectroscopic FTIR analysis will be used to investigate interactions and conformational changes on the aggregates obtained at different growth conditions. We will report both the attainment of biologically active aggregates showing amidase activity and the success in aggregates solubilization using L-arginine resulting in amidase recovery from the aggregates.

2 Materials and methods

2.1 Chemicals

Triptone and yeast extract, constituents of the culture medium LB were obtained by Oxoid Ltd. (Cambridge, England). Ampicillin, IPTG, urea, L-arginine, Sepharose 6B, Blue Coomassie G 250, and molecular mass markers were purchased from Sigma–Aldrich, Inc (St. Louis, USA). Acetamide, $FeCl_3$, and $AgNO_3$ were acquired in Merck, hydroxylamine in Fluka (St. Gallen, Switzerland) and Tris-HCl in Riedel–deHaën (Seelze, Germany). Ultrafiltration P-10 membranes were obtained from Millipore (Massachusetts, USA).

2.2 Strain and culture conditions

A recombinant strain JM109 of *E. coli* containing the wild-type amidase gene *amiE* of strain 8602 of *P. aeruginosa* was used as the source of enzyme [29]. Recombinant *E. coli* JM 109 cells were grown at different conditions in LB medium supplemented with 100 μg/mL ampicillin. The parameters tested were two different incubation temperatures 37°C or 25°C, different IPTG concentrations for induction ranging from 0.1 to 1 mM and medium supplementation with 3% v/v ethanol.

2.3 Soluble (S) and insoluble (IS) cell fraction preparation

Cells grown at different culture conditions were collected by centrifugation, washed, and frozen when the absorbance of the culture at 600 nm was ~1 or higher (stationary phase). Same amount of frozen cells were harvested for all the conditions, in particular frozen cells of each culture condition were ressuspended in 10 mM sodium phosphate buffer, pH 7.2 (30 mg/mL) and broken by an optimized sonication step with three bursts of 5 s at 150 W in an iced bath. Part of this suspension (15 mg/mL), which represents total cell extract (T), was collected and the remaining was centrifuged at $9840 \times g$ for 30 min at 4°C in order to separate soluble (S) and insoluble (IS) cell fractions. IS fractions were previously washed in order to exclude soluble protein trapped in the IS fractions and ressuspended in buffer to achieve the same volume as the respective S fraction. Cell fractions (T, S, and IS) of the tested culture conditions were assayed for amidase activity and protein content analyzed by gel electrophoresis as described in Section 2.4, total protein concentration was determined using Bradford assay [30] and subsequently lyophilized.

2.4 Amidase activity assay and gel electrophoresis analysis

Amidase acyl transferase activity was determined by using a previously reported [31] spectrophotometric assay for the detection of the formation of acetohydroxamic acid using acetamide and hydroxylamine as substrates. This product was detected through the addition of $FeCl_3$ 6% w/v in HCl 2% w/v to the reaction mixture and the formation of a brown color was quantified at 490 nm ($\varepsilon = 0.026\ mM^{-1}/cm^{-1}$). One enzyme unit (U) is defined as the amount of enzyme required to hydrolyze 1 μmol of acetamide per min at 25°C under these experimental conditions.

Native PAGE and SDS-PAGE with 8 M urea of T, S, and IS cell fractions for all the tested conditions were carried out as described elsewhere [32] and stained for protein with silver nitrate [33]. For fast detection of amidase presence in the lanes of the electrophoresis gels, the detection of amidase activity *in situ* in native PAGE was carried out as described previously [29, 34, 35]. Briefly, the gels were incubated at 25°C during 15 min in the presence of acetamide and hydroxylamide, the amidase reaction substrates [36]. The formation of the product, acetohydroxamic acid, in the gels can be detected by contact of the gel with a soaked filter paper with $FeCl_3$ 6% w/v in HCl 2% w/v with the formation of a brown color. The color change indicates the presence of biologically active amidase in the analyzed cell fractions. A sample of solution containing purified amidase was used as control.

2.5 Extraction and purification of recombinant amidase

Amidase was purified from cell-free extract by affinity chromatography on epoxy-activated Sepharose 6B-acetamide column followed by gel filtration chromatography on Sephacryl S-300HR column as described previously (13 U/mg protein and purification factor of 9) [29]. Subsequently, the purified amidase solution was lyophilized.

2.6 Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy was used to evaluate the expression of amidase in the soluble (S) and insoluble (IS) fractions obtained in Section 2.3 for the different growth conditions. KBr pellets of the S and IS fractions were prepared and infrared spectra were recorded using a Tensor 27 Bruker (Madison, USA) FTIR spectrometer by averaging 400 scans in the spectral range of 400–4000 cm^{-1} at 4 cm^{-1} resolution to obtain spectra with a high ratio signal/noise (*S/N*). A similar approach was used for KBr pellets of the purified amidase obtained in Section 2.5. The OPUS^{NT} was used for the acquisition and edition of the FTIR spectra.

2.7 Solubilization of amidase aggregates in IS fractions

The ability of L-arginine and chaotropic agent guanidine hydrochloride (GdnHCl) in the successful solubilization of amidase aggregates was evaluated. The IS cell fractions obtained for the different growth conditions were suspended in concentrations, ranging from 0 to 1.5 M, of solubilizing agent such as L-arginine or GdnHCl. Each mixture was incubated at room temperature with orbital shaking for 24 h. After such period the sample was extensively washed with 10 mM sodium phosphate buffer, pH 7.2, and centrifuged at 9840 × *g* for 30 min at 4°C in order to eliminate residues of the solubilizing agent. For each condition R and NR cell fractions were obtained after solubilization corresponding, respectively, to the enzyme which was recovered in the soluble form and the non-recovered enzyme in the insoluble fraction after centrifugation. The R and NR fractions were assayed for amidase transferase activity and gel electrophoresis analysis as described in Section 2.4, total protein concentration was determined using the Bradford assay [30] and subsequently lyophilized.

3 Results and discussion

3.1 Effect of growth conditions in amidase production

In order to determine the influence of parameters such as growth temperature, IPTG concentration, and ethanol shock in the production of amidase, recombinant *E. coli* JM 109 cells containing the wild-type amidase gene from *P. aeruginosa* were grown either at 37 or 25°C in LB medium which was supplemented or not with 3% v/v ethanol. The synthesis of the enzyme was also induced by addition of different concentrations of IPTG to the culture media during the middle exponential phase. Culture cells were harvested at stationary phase, disrupted and S and IS cell fractions were obtained as described in Section 2.3.

Table 1 summarizes the results of the growth rate constants obtained at the different growth conditions described in Section 2.2 and for each of the prepared cell fractions (S and IS) the protein and amidase yield. Figure 1 summarizes the results in enzyme specific activity in the S and IS fractions obtained from cells subjected to the different growth conditions.

In Table 1 (column 3), it is noticeable an overall increase in the protein yield in the S cell fraction at 25°C and particularly at each condition with ethanol supplementation. The reduction of the cultivation temperature and limiting on the gene expression induction, by the inducer concentration such as IPTG, are reported to increase the amount of soluble protein [15, 37–40]. Thomas and Baneyx [17] have also reported that supplementation of the grown medium with 3% v/v ethanol improved the solubility of some recombinant proteins. The highest yield in soluble protein was obtained for the

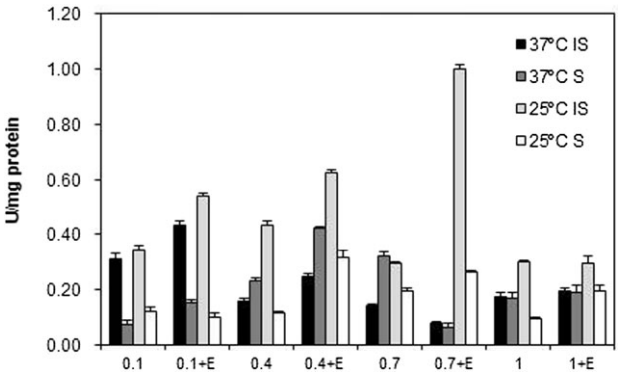

Figure 1. Effect of temperature, IPTG concentration (0.1–1 mM) and 3% v/v ethanol supplementation (+E) on amidase specific activity obtained in the soluble (S) and insoluble (IS) fractions of cells submitted to conditions described in Section 2.2. Data are representative of three independent experiments.

growth condition of 0.4 mM IPTG at 25°C with ethanol.

On the contrary it was verified, for the growth conditions at 25°C, a decrease in amidase yield in the S cell fractions (Table 1, column 5) and that the enzyme aggregated substantially in a biologically active form at these IS fractions (Table 1, column 6). In this case, the higher values of soluble amidase specific activity and yield were obtained for conditions at 37°C. As can be seen in Fig. 1, for the S fractions, the highest amidase specific activity was obtained for cells grown at 37°C in ethanol presence with 0.40 mM IPTG induction.

Additionally, the results shown in Fig. 1 and Table 1 (column 6) clearly demonstrated that all the conditions in the IS cell fractions contained biologically active amidase. In the case of IS cell fractions, a higher specific activity and yield of aggregated amidase was obtained for conditions at suboptimal conditions 25°C relatively to conditions at 37°C. As can be seen in Fig. 1, for all the tested growth conditions and obtained cell fractions the maximum specific activity was found in the IS cell fraction at 25°C with ethanol and 0.7 mM IPTG.

Considering the ethanol effect on the enzyme yield, cells grown in ethanol presence produced the maximum specific activity of soluble (S) and insoluble (IS) amidase as seen in Fig. 1. It becomes evident that the cultures submitted to ethanol shock, demonstrated an overall increase in the activity of recombinant amidase.

Figure 2 shows the SDS-PAGE analysis of the produced proteins in T, S, and IS cell fractions of the cultures induced with 0.7 mM IPTG subjected to different growth conditions, as representative of identical profile obtained when other IPTG concentrations were used. The insoluble fractions at 25°C always showed the presence of amidase as the main component of all the protein which accumulated in the cell in the form of insoluble aggregates (Lane 25°C +E; IS and Lane 25°C; IS) as opposed to the conditions at 37°C in which others proteins ex-

Table 1. Effect of temperature, IPTG concentration (0.1–1 mM) and 3% v/v ethanol supplementation (+E) in the yield of protein and amidase specific activity in soluble (S) and insoluble fractions (IS) from *E. coli* pKK WT cells in LB ampicillin supplemented media submitted to conditions described in Section 2.2

Temperature	[IPTG] (mM)	Protein (mg) (protein yield %)		Amidase specific activity (U/mg protein) (amidase yield %)	
		S	IS	S	IS
37°C	0.1	0.251 (46.3)	0.291 (53.7)	0.085 (19.1)	0.361 (80.9)
	0.1 +E	0.381 (64.8)	0.207 (35.2)	0.142 (24.3)	0.443 (75.7)
	0.4	0.463 (53.8)	0.398 (46.2)	0.235 (58.5)	0.167 (41.5)
	0.4 +E	0.445 (48.3)	0.477 (51.7)	0.421 (61.7)	0.261 (38.3)
	0.7	0.781 (70.6)	0.325 (29.4)	0.324 (69.4)	0.143 (30.6)
	0.7 +E	0.507(77.1)	0.151 (22.9)	0.094 (49.7)	0.095 (50.3)
	1	0.712 (69.2)	0.317 (30.8)	0.183 (50.6)	0.179 (49.4)
	1 +E	0.421 (69.1)	0.188 (30.9)	0.197 (50.6)	0.192 (49.4)
25°C	0.1	0.678 (76.0)	0.214 (24.0)	0.137 (29.3)	0.331 (70.7)
	0.1 +E	0.632 (74.8)	0.213 (25.2)	0.118 (17.9)	0.543 (82.1)
	0.4	0.483 (56.7)	0.369 (43.3)	0.126 (22.1)	0.445 (77.9)
	0.4 +E	0.731 (80.0)	0.183 (20.0)	0.314 (33.5)	0.623 (66.5)
	0.7	0.703 (75.9)	0.223 (24.1)	0.197 (39.0)	0.308 (61.0)
	0.7 +E	0.582 (74.8)	0.196 (25.2)	0.283 (21.9)	1.012 (78.1)
	1	0.219 (43.4)	0.298 (56.6)	0.093 (24.2)	0.307 (76.8)
	1 +E	0.831 (69.6)	0.363 (30.4)	0.194 (38.5)	0.310 (61.5)

Data are representative of three independent experiments.

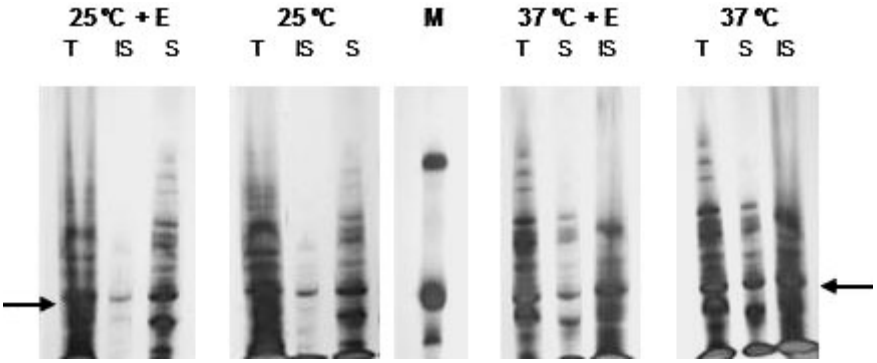

Figure 2. SDS-PAGE analysis using a 10% separating gel with 8 M urea of the *E. coli* pKK WT cell fractions obtained as described in Section 2.3: total cell extract (T), insoluble fraction (IS), and soluble fraction (S). The lanes represent the protein profile of the cell fractions for different growth condition in LB ampicilin medium using 0.7 mM IPTG: 25°C with 3% v/v ethanol supplementation (25°C +E), 25°C, 37°C with 3% v/v ethanol supplementation (37°C +E), and 37°C. M-protein markers: BSA, ovalbumin, α-chymotripsin, and lisozyme (66, 45, 24.5, and 14.3 KDa) from top to bottom. The position of amidase subunits is indicated by arrows. Data are representative of three independent experiments.

pressed by the strain also aggregated (Lane 37°C +E; IS and Lane 37°C; IS). This was also verified in Fig. 3 native gel gB (Lane 25°C +E; IS and Lane 25°C; IS). Both observations indicate that the IS fractions obtained for conditions at 25°C contain amidase as the major protein component, corroborating the high values of specific activity obtained in Fig. 1. This may support a simplified enzyme purification protocol from the latter aggregates. The presence of amidase subunits was identified through its molecular weight, ~38 KDa [41] corresponding to the arrow shown in Fig. 2. In order to confirm the electrophoretic profile of the samples and the presence of amidase activity in the S and IS cell fractions obtained from each condition, amidase transferase activity was performed in situ in a native PAGE as described in Section 2.4. Results are presented in Fig. 3. The development of a brown color in the lanes from the filter paper fA and fB when compared with a sample of purified amidase C, allowed the validation of amidase presence and biological activity in the cell fractions from every growth condition. In the filter paper the intensity of the formed color in the top of fA and fB revealed to be comparative to the previously assayed enzymatic activity of the fractions, with fractions obtained from conditions at 25°C (fB) demonstrating a more intense color than fractions from conditions at 37°C (fA). Again the highest amidase specific activity was obtained for the cells grown at 25°C with ethanol and induced with 0.7 mM IPTG (fB-Lane 25°C +E; IS). The native gels gA and gB bands,

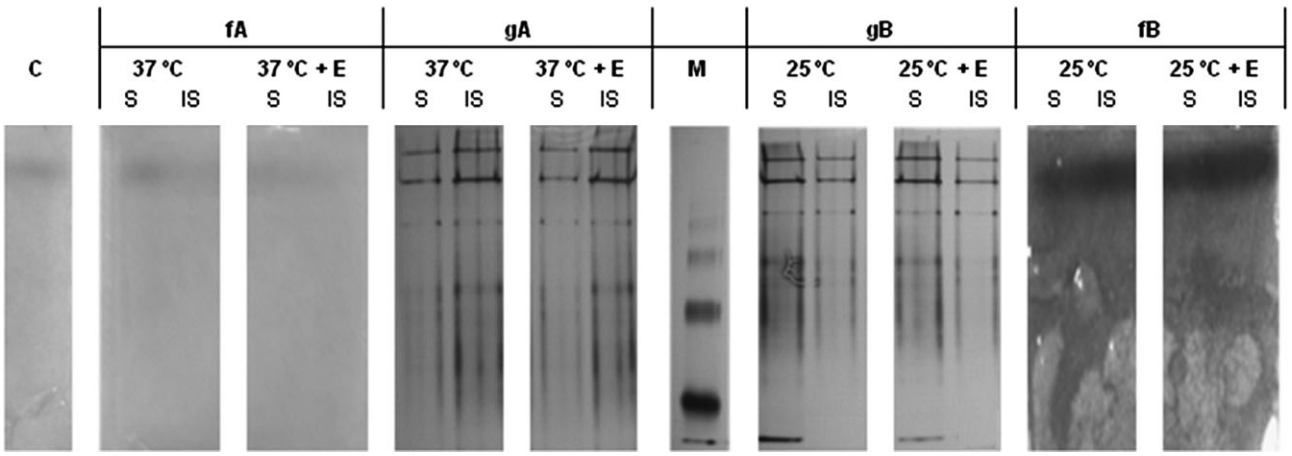

Figure 3. Native PAGE analysis using a 7.5% separating gel of the *E. coli* pKK WT cell fractions, obtained as described in Section 2.3 at different growth conditions (gA and gB). Filter paper showing the detection of amidase activity *in situ* as described in Section 2.4 (fA e fB). The lanes represent purified native amidase as described in Section 2.5 (C) insoluble (IS) and soluble cell fraction (S) from different growth condition in LB ampicilin medium using 0.7 mM IPTG: 25°C with 3% v/v ethanol supplementation (25°C +E), 25°C, 37°C with 3% v/v ethanol supplementation (37°C +E) and 37°C. M-protein markers: 196, 132, 66, 45, 17.8, and 14.3 KDa from top to bottom. Data are representative of three independent experiments.

which were stained for protein with silver nitrate, were found to be coincident with the brown color in the filter papers fA and fB, respectively, demonstrating the position of the amidase band near the protein marker at 196 KDa.

3.2 Structural analysis by FTIR of the IS fractions of the different growth condition

The conformational status of the amidase aggregates obtained at different growth conditions was examined by FTIR spectroscopy. Analysis of the FTIR spectra in the amide I region between 1700 and 1500 cm^{-1} is a common technique to examine bacterial insoluble aggregates of produced proteins, since this region provides quantitative and qualitative relationships between the various secondary structures present in proteins [42]. Figure 4 represents FTIR spectra in the amide I region of the IS cell fractions grown in the presence of ethanol at 25°C (dashed line) and 37°C (dotted line). These FTIR spectra are representative of the spectra obtained for the IS fractions of all the growth conditions. Figure 4 also exhibits the absorbance FTIR spectra in the amide I region of a native purified amidase (full line) obtained after a purification step described in Section 2.5. The observation of the represented spectra reveals a similar amide I band centered at 1657 cm^{-1} corroborating amidase presence in the obtained IS fractions as established in Fig. 2. In all the obtained FTIR spectra a linear baseline was visible between 1800 and 2000 cm^{-1} which suggested minor interferences and was used as criteria for spectral correction [43]. Furthermore, in order to avoid misinterpretation of results obtained from the FTIR spectra resulting from possible artifacts such as water interference or KBr sampling, several approaches for the analysis of the spectra were applied [44–46]. In order to clarify the analysis, the second derivative spectra were represented of the IS fractions at different growth conditions and compared with the spectrum of the purified amidase (Fig. 5). All second derivative spectra displayed one major component of the amide I band at 1657 cm^{-1} which can be assigned to α-helix structure. In the second derivative spectrum of the purified enzyme, bands around 1628 and 1637 cm^{-1} were observed which can be assigned to the native antiparallel and parallel β-sheet structures, respectively [47]. In the spectra of the aggregated amidase in the IS fractions of all the growth conditions, a single band was observed in this region, around 1632–1636 cm^{-1}, suggesting that in the aggregated state a disorganization of the native β-sheet structures occurred into a new arrangement with formation of intermolecular interactions. This observation was supported by the presence of an intense band at ~1680 cm^{-1} in the second derivative spectra of all the IS cells fractions [48]. Another approach was the estimation of the width at medium height for the amide I band of the obtained FTIR spectra. In this case the width at medium height of the amide I band was higher for all the spectra from IS cell fractions when compared with the purified amidase spectrum (data not shown). Again, such results suggest an increase in the intermolecular interactions for the IS cell fractions comparatively to the purified enzyme, as reported previously [49].

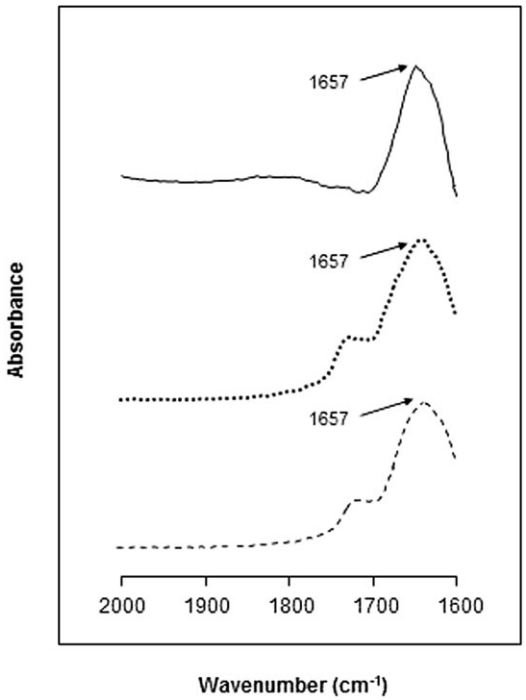

Figure 4. Comparison of FTIR spectrum in amide I region of the purified amidase (full line) and IS fractions of the following growth conditions: 0.1 mM IPTG + 3% v/v ethanol at 37°C (dotted line) and 0.7 mM IPTG + 3% v/v ethanol at 25°C (dashed line). Data are representative of three independent experiments.

The estimation of the ratio between the absorbance at 1620–1640 cm^{-1} (intermolecular β-sheet) and 1650–1658 cm^{-1} (α-helix), for the second derivative spectra of the IS fractions at different growth conditions, allowed the evaluation of the contribution of these components for the amidase aggregated structure. The ratios obtained were compared with the ratio for purified amidase second derivative spectrum, between the absorbance of the intramolecular β-sheets at 1637 cm^{-1} and α-helices at 1657 cm^{-1}. The obtained ratios are represented in Fig. 6. In general, the results demonstrated that the ratios from aggregated amidase in the

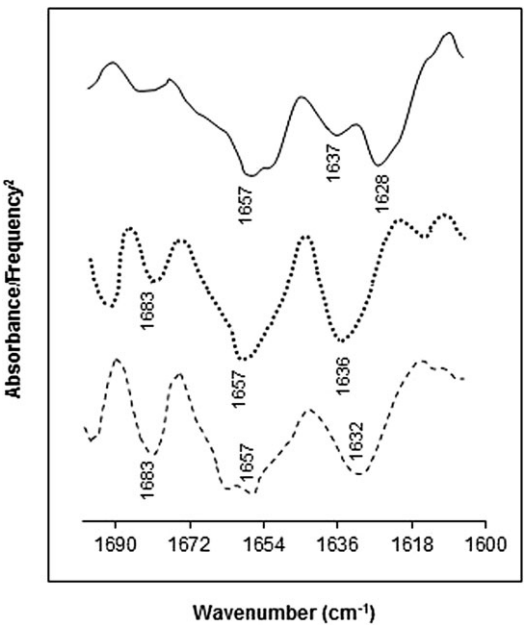

Figure 5. Comparison of second derivative FTIR spectrum in amide I region of the purified amidase (full line) and IS fractions of the following growth conditions: 0.1 mM IPTG + 3% v/v ethanol at 37°C (dotted line) and 0.7 mM IPTG + 3% v/v ethanol at 25°C (dashed line). Data are representative of three independent experiments.

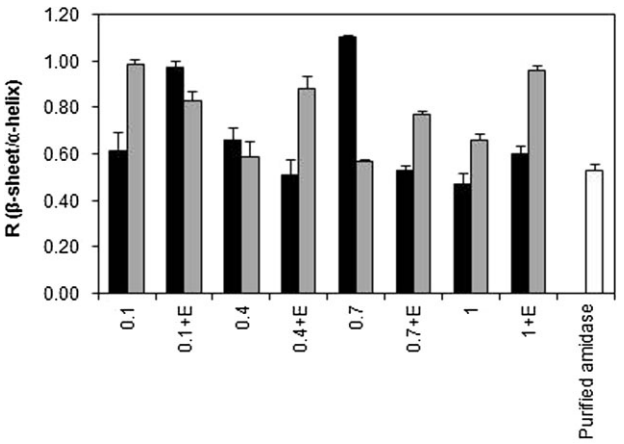

Figure 6. Ratio of absorbance values between the bands 1620–1640 cm^{-1} and 1650–1658 cm^{-1} of the second derivative FTIR spectrum of the fraction of IS different growth conditions, for temperatures of 25 and 37°C (gray and black bar, respectively), different concentrations of IPTG (0.1–1 mM) and presence of 3% v/v ethanol (+E), and the solution of purified amidase (white bar). Data are representative of three independent experiments.

IS fractions of the different growth conditions (black and gray bars in Fig. 6) are higher than the ratio from the purified enzyme (white bar in Fig. 6). These results suggest that the amidase in the IS fractions presented higher proportion of aggregated β-sheets comparatively to the purified enzyme, as demonstrated for other aggregated proteins [15, 50]. In addition, the ratio obtained in IS fractions for growth conditions of 37°C (Fig. 6 black bars) are overall lower than the ratio from the IS fractions of conditions at 25°C (Fig. 6 gray bars), mainly in ethanol presence. Suggesting a lower content of aggregated β-sheets in the former relatively to the IS fractions from conditions at 25°C. Hence, the growth conditions at 25°C in ethanol presence demonstrated overall a higher content of aggregated β-sheets in the IS fractions with higher enzymatic activity, as previously mentioned. This observation was confirmed by band shifts in the second derivative spectra of the IS fractions of the aggregated β-sheets in Fig. 5. As can be seen in Fig. 5, the formation of aggregates leaded to a down shift in the band peak positions of the intramolecular β-sheets in the spectrum of native purified amidase from 1637 to 1636 and 1632 cm^{-1} in the spectra of the IS fractions. In conditions at 37°C the band is positioned at 1636 cm^{-1} and in conditions at 25°C in ethanol at 1632 cm^{-1}, with a more intense band at 1683 cm^{-1} in the later, both suggesting more hydrogen bonded aggregated β-sheets in the IS fractions with stronger interactions in conditions at 25°C in ethanol [44, 48, 51]. These results were also verified in the amide II region of the spectra.

3.3 Analysis of the solubilization of the amidase aggregates in IS fractions

Several authors [22–25] in the last years have stated that protein insoluble aggregates could be easily solubilized in non-denaturating conditions avoiding strong denaturation. In our case it seemed appropriated to test this approach as the structural and biological properties of the obtained aggregates revealed biologically active aggregates in all the tested growth conditions some of which demonstrated lower content of intermolecular interactions. The IS cell fractions obtained for each growth condition were subjected to non-denaturing solubilization using different concentrations of L-arginine. This procedure was compared with solubilization using a selected chaotropic agent such as GdnHCl, as described in Section 2.7. After solubilization the obtained R and NR fractions were characterized.

Figure 7 represent the ratio in the R fractions between amidase activity measured after and before solubilization of the different IS fractions. Observation of Fig. 7 revealed that in all the solubilized IS cell fractions the use of 0.25, 0.5, and 1 M L-arginine in the solubilization results in the highest yield of recovery of amidase in a bioactive

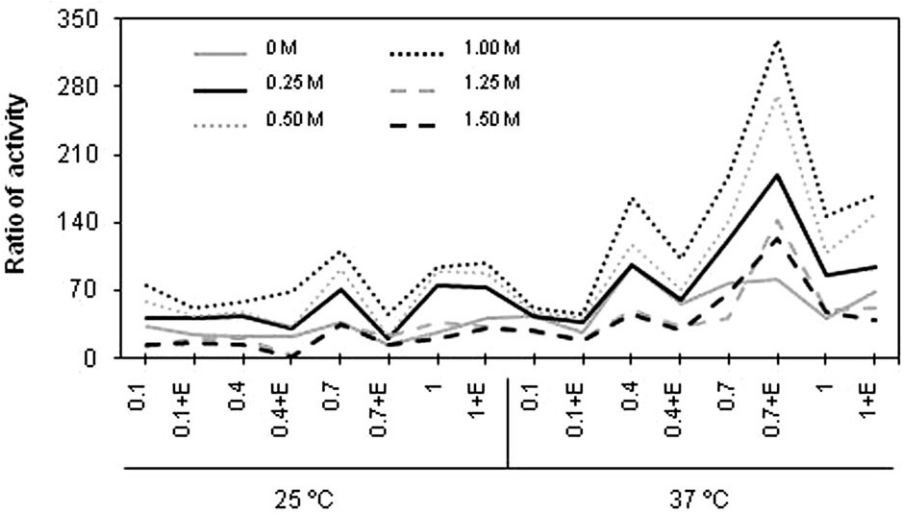

Figure 7. Ratio between the enzyme activity of the R (recovered) fractions obtained after solubilization with different concentrations of L-arginine 0 M (gray full line), 0.25 M (black full line), 0.5 M (gray dotted line), 1 M (black dotted line), 1.25 M (gray dashed line), and 1.5 M (black dashed line), and the specific activity of enzyme present in the insoluble fraction (IS) of different growth conditions, for temperatures of 25 and 37°C, different concentrations of IPTG (0.1–1 mM) and presence of 3% v/v ethanol (+E). Data are representative of three independent experiments.

and soluble form. The best results in solubilization were achieved using 1 M L-arginine (top black dotted line in Fig. 7) with the highest ratios in all the R fractions recovered. However, for high concentrations of L-arginine in solubilization, such as 1.25 or 1.5 M (gray and black dashed line in Fig. 7), there was a lower ratio of enzyme recovery probably due to strong interactions between L-arginine high concentrations and the aggregates which might limit the enzyme solubilization, as reported in Ref. [52]. In solubilization using the highest concentrations of L-arginine 1.25 and 1.5 M there was verified a high yield of enzyme activity in the aggregated form remaining in the NR fractions (data not shown) contrary to the lowest concentrations of L-arginine, 0.25, 0.5, and 1 M, which as stated favored the recovery of the enzyme in the soluble form in the R fractions. After solubilization of all IS fractions, both R (Fig. 7) and NR fractions exhibited small increases in the amidase activity in the absence of L-arginine (0 M). This suggests the importance of agitation in the solubilization of aggregated enzyme and corroborates the fragility of the interactions responsible for enzyme aggregation especially in aggregates obtained at 37°C. It was noticeable at Fig. 7 that the IS fractions obtained from growth conditions at 37°C (right side of Fig. 7) demonstrated higher yields in solubilization and enzyme recovery comparatively to the aggregates obtained at 25°C (left side of Fig. 7). The highest yield in the recovery of the soluble enzyme was obtained for the IS fraction of the growth condition at 37°C using 0.7 mM IPTG and ethanol with a recuperation of ~329-fold of amidase specific activity in the soluble R fraction using 1 M L-arginine in solubilization. L-Arginine reported capacity to weaken intermolecular interactions responsible for aggregation [22, 24, 53] clarified this results. In fact, in aggregates obtained at 37°C, which demonstrated lower content of intermolecular interactions, it was confirmed higher yields of enzyme recovery comparatively to the enzyme recovery from aggregates obtained at 25°C. As mentioned above the latter contain stronger interactions.

As opposed to the results obtained with L-arginine there was lower or no amidase specific activity in R and NR fractions obtained after solubilization using chaotropic agent GdnHCl possibly due to its denaturing properties [54] and the incapacity for the enzyme to renaturate into its quaternary structure and to recover its activity. The above reported results were confirmed by the observation of the electrophoretic profile of the R and NR fractions obtained after solubilization of the aggregates.

4 Concluding remarks

The present study reports on *P. aeruginosa* amidase aggregation in recombinant *E. coli*. Our results demonstrated that variations in different parameters could not prevent aggregates production, in all the tested growth conditions, and amidase was identified in the aggregates. Nevertheless, the higher amidase activity in the soluble fraction was

obtained in growth conditions at 37°C indicating that these conditions favored the production of soluble enzyme. Ethanol supplementation and 25°C temperature favored amidase high yield and an increase in amidase activity, however in an aggregated form. Our results showed that, the biological activity of the insoluble fractions obtained was comparable or in most cases higher than verified in the soluble fractions.

A method was employed for the solubilization using non-denaturing conditions of the aggregated amidase using L-arginine. This solubilization step avoided the registered inactivation when using chaotropic agents such as GdnHCl. The growth conditions at 37°C, which produced insoluble aggregates with lower intermolecular interactions registered by FTIR, demonstrated a facilitated recovery of the enzyme in the solubilization step under non-denaturing conditions. The higher interactions observed in aggregates obtained at 25°C with ethanol supplementation, compromised the recuperation by solubilization under these conditions. However, the specific activity of the extracted amidase was successfully enhanced after the performed simple procedure of solubilization using L-arginine.

In conclusion, to the authors' knowledge insoluble aggregates of recombinant *P. aeruginosa* amidase were never reported nor characterized. From a practical point of view, the identified amidase is biologically active and present in the insoluble aggregates might possibly be used effectively as catalysts for industrial applications without any preceding protein-refolding step. Nevertheless, a simple solubilization procedure was illustrated in this work allowing the efficient recovery of the enzyme from the aggregates with an enhanced activity.

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