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Identification of *Escherichia coli* K12 YdcW protein as a γ-aminobutyraldehyde dehydrogenase

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Abstract γ-Aminobutyraldehyde dehydrogenase (ABALDH) from wild-type *E. coli* K12 was purified to apparent homogeneity and identified as YdcW by MS-analysis. YdcW exists as a tetramer of 202 ± 29 kDa in the native state, a molecular mass of one subunit was determined as 51 ± 3 kDa. K_m parameters of YdcW for γ-aminobutyraldehyde, NAD^+ and $NADP^+$ were 41 ± 7, 54 ± 10 and 484 ± 72 μM, respectively. YdcW is the unique ABALDH in *E. coli* K12. A coupling action of *E. coli* YgjG putrescine transaminase and YdcW dehydrogenase in vitro resulted in conversion of putrescine into γ-aminobutyric acid.

Keywords: Putrescine degradation; γ-Aminobutyraldehyde dehydrogenase; Putrescine aminotransferase; *Escherichia coli*

1. Introduction

Putrescine is present in virtually all living cells, from bacteria to plant and human cells and is essential for cell structure and physiology [1,2]. It can bind to nucleic acids, stabilize membrane and stimulate activity of several enzymes [2–4]. The intracellular putrescine deficiency leads to poor cell growth rate and poor survival [5]. On the other hand, despite the proved necessity of intracellular polyamine for cellular metabolism, polyamine accumulation can lead to inhibition of cellular growth and protein synthesis [5,6]. Thus, it is clear that the cell mechanisms for maintenance of the optimum intracellular putrescine contents at normal environment as well as at stress conditions should exist. According to the current data, steady state concentration of the intracellular putrescine in *Escherichia coli* is generally mediated by specific influx/efflux transport systems [7–9] and by coupling action of putrescine synthesis from arginine or ornithine [10] and its degradation. There are two routs of putrescine degradation to γ-aminobutyric acid (Fig. 1) via arginine decarboxylase pathway [11], requiring putrescine:α-ketoglutarate aminotransferase (PAT) and γ-aminobutyraldehyde dehydrogenase (ABALDH), and via γ-glutamylated intermediates pathway [12].

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Abbreviations: ABALDH, γ-aminobutyraldehyde dehydrogenase; ABAL, γ-aminobutyraldehyde; PAT, putrescine:α-ketoglutarate transaminase; GABA, γ-aminobutyric acid

Previously, it was showed that the PLP-dependent PAT in *E. coli* K12 is encoded by *ygjG* gene [13]. The ABALDH was purified from *E. coli* mutants and characterized about 20 years ago [14], but the corresponding gene has not been determined yet. The goal of this work was identification of the gene encoding ABALDH in the wild-type *E. coli* K12 strain.

2. Materials and methods

2.1. Bacterial strains

E. coli K12 MG1655 (K12 λ-, F-) strain was used for ABALDH purification. Strain MG1655 ΔydcW::kan was obtained according to the method described by Datsenko and Wanner [15].

2.2. Chemicals

γ-Aminobutyric acid (GABA), putrescine, butyraldehyde, NAD^+, $NADP^+$ and γ-aminobutyraldehyde-diethyl acetal were obtained from Sigma–Aldrich Corporation (USA). γ-Aminobutyraldehyde (ABAL) was synthesized according to the method described by Matsuda and Suzuki [16]. Acetonitrile was purchased from Kriochrom (Russia). All solutions were prepared using Milli-Q water, standard solutions were stored at 4 °C.

2.3. Cell growth and preparation of cell-free extracts

Strains were routinely grown aerobically at 37 °C in LB broth or M9 salts medium [17] without ammonium sulfate supplemented with one of the carbon sources at 0.4% (glucose, glycerol, putrescine) and one of the nitrogen sources at 0.2% (ammonium sulfate, putrescine). For induction of ABALDH activity cells of MG1655 strain were grown overnight in M9 medium, washed by 15 mM NaCl and diluted to OD_{600} of 0.7 by LB, M9 and M9 nitrogen (or carbon) limited mediums. The resulting cultures were cultivated during 5 h.

The procedure of cell-free extracts preparation was carried out as previously described [13] using 50 mM potassium phosphate buffer, pH 7.4. Proteins of cell-free extract were precipitated by ammonium sulfate (85% of saturation), resuspended in the same buffer and desalted by gel-filtration using PD-10 Desalting column (Amersham Bioscience, UK).

2.4. Enzyme assay

NAD^+-dependent ABALDH activity of partially purified enzyme and in cell-free extracts was assayed by HPLC-analysis of the time-depending GABA formation. The assay mixture (final volume 0.03 ml) containing 0.1 M Tris–HCl buffer, pH 7.5, 1 mM NAD^+, 1 mM ABAL and 6.5 or 19.5 μg of enzymatic extracts incubated at 37 °C. Putrescine aminotransferase reaction was carried out with purified recombinant YgjG enzyme as described previously [13]. Coupling reaction of purified YdcW and YgjG was assayed with 10 mM putrescine, 25 μM PLP and 1 mM NAD^+ in 0.1 M sodium citrate buffer, pH 5.5, at 37 °C.

The enzymatic activity of purified ABALDH was determined spectrophotometrically by measuring the absorbance increase at 340 nm (Shimadzu spectrophotometer UV1240) at 25 °C. The

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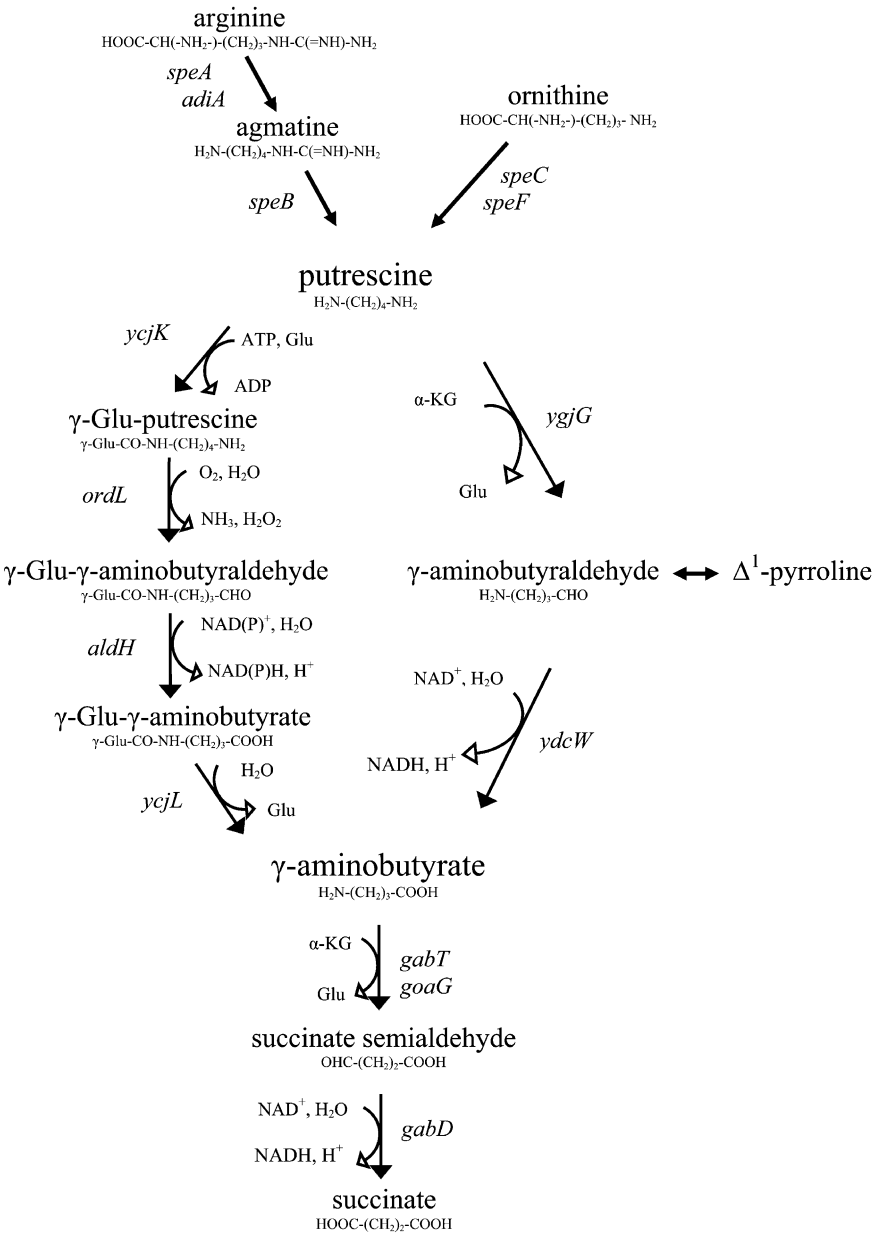

Fig. 1. Schematic presentation of putrescine biosynthesis and catabolism in *E. coli*. Details of the pathways are given elsewhere [12,26]. The *ygjG* gene encodes putrescine aminotransferase (EC 2.6.1.29) [13]. The *ydcW* gene as reported in this study encodes ABAL dehydrogenase (EC 1.2.1.19).

reaction was initiated with the addition of substrate to the 0.4 ml assay mixture described above supplemented with 0.15–1.0 μg of the enzyme. The absorbance change was linear for at least 5 min. The kinetic analysis of YdcW was carried out by varying the concentration of coenzyme (NAD^+ or $NADP^+$) from 0.025 to 5 mM at ABAL concentration of 0.5 mM and by varying the substrate (ABAL or butyraldehyde) concentration from 0.025 to 10 mM at NAD^+ concentration of 0.5 mM. In the case of NAD^+ the maximum ABALDH activity value was attained for concentration of ABAL about 0.3 mM. Inhibition of excess substrate was observed at concentration superior to 1 and 4 mM for ABAL and butyraldehyde, respectively (data not shown). The K_m and V_{max} parameters with standard errors for ABAL and coenzymes were determined from Michaelis–Menten kinetic equation plots obtained by means of non-linear regression of the experimental points (SigmaPlot 8.0, http://www.systat.com/products/SigmaPlot). The k_{cat} and k_{cat}/K_m values were calculated according to the molecular mass of the YdcW is equal to 50.83 kDa.

2.5. HPLC-analysis

The GABA, ABAL and L-glutamic acid formation were measured by HPLC-analysis using high pressure chromatograph (Waters, USA) with fluorescence scanning detector 1100 series (Agilent, USA). The excitation wavelength was 250 nm, the range of emission wavelengths was 320–560 nm. The separation by Accq-tag method was performed in the column Nova-Pak™ C18 150 × 3.9 mm, 4 μm (Waters, USA) at 37 °C using concentrated Accq-tag Eluent A (Waters, USA). Injection volume was 5 μl. Formation of amino acid derivatives with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate was obtained using Accq-Fluor™ kit (Waters, USA) and their separation was performed according to Waters manufacturer's recommendation.

2.6. Purification of ABALDH from E. coli MG1655 strain

ABALDH was purified from *E. coli* MG1655 strain using the following procedures. *Step 1*: A 10 ml of overnight culture MG1655 was used to inoculate 100 ml LB-broth in 1 l flask. Cells were culti-

Table 1
Induction of ABALDH activity in *E. coli* K12 MG1655 strain depending on different growth conditions

Growth conditions[a]	I	II	III	IV	V	VI
Carbon source	LB	Gluc	Glyc	Ptr	Gluc	Glyc
Nitrogen source		NH_4^+	NH_4^+	NH_4^+	Ptr	Ptr
ABALDH activity (nmol/min/mg)	25	5.2	16.5	17.1	20.6	40.1

Abbreviations: LB, LB-broth; Ptr, putrescine; Gluc, glucose; Glyc, glycerol; NH_4^+, $(NH)_2SO_4$.
[a]Growth conditions described in Section 2.

Table 2
Purification of the ABALDH from *E. coli* K12 MG1655

Purification step	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Yield (%)	Purification fold
Cell extract	187.20	4.493	0.024	100.0	1
Ammonium sulfate (60–85%)	9.60	4.426	0.461	98.6	19
Anion-exchange chromatography	1.08	3.054	2.828	68.0	117
Hydrophobic interaction chromatography	0.08	0.678	8.478	15.1	353

vated at 37 °C for about 12 h and then harvested by centrifugation, washed by 100 mM NaCl solution, and stored at −70 °C until used. *Step 2*: Frozen cells (1.8 g) were thawed, suspended in 24 ml of buffer A (50 mM potassium phosphate, pH 7.4, 1 mM DTT) and disrupted by two passages through French pressure cell (Thermo Spectronic; $P = 2{,}5$ Psi) followed by centrifugation to remove debris. *Step 3*: Cell-free xtract was fractionated with ammonium sulfate. Precipitate (60–85% of saturation) was dissolved in 24 ml of buffer A and desalted by gel-filtration on the Sephadex G-25 column (2.5×13 cm) equilibrated with the same buffer. Volume of the final protein preparation was about 30 ml. *Step 4:* Anion-exchange FPLC was carried out using ÄKTAbasic100 system supplemented with Source15Q column (Amersham Pharmacia Biotech, UK). 10 ml of the protein preparation obtained from *Step 3* was applied to the column equilibrated with buffer A. The elution was carried out at flow rate 1 ml/min by linear stepwise gradient with: (1) five column volumes gradient from 0 to 0.05 M NaCl in buffer A; (2) 20 column volumes gradient from 0.05 to 0.3 M NaCl in buffer A; (3) five column volumes gradient from 0.3 to 0.5 M NaCl in buffer A. 2 ml fractions were collected. The peak containing ABALDH activity was eluted from 175 to 195 mM NaCl concentrations. This procedure was repeated three times. Active fractions were pooled and desalted by gel-filtration on the Sephadex G-25 column (2.5×13 cm) equilibrated with buffer A. *Step 5:* Hydrophobic interaction chromatography was carried out using ÄKTAbasic100 system supplemented with Resource PHE columns (Amersham Pharmacia Biotech, UK). Ammonium sulfate was added to the 10 ml of protein preparation obtained from *Step 4* to final concentration of 1.5 M. Protein solution was applied to the column equilibrated with buffer B (50 mM potassium phosphate, pH 7.4, 1 mM DTT) supplemented with 1.5 M ammonium sulfate. The elution was carried out at flow rate 1 ml/min by linear stepwise gradient with: (1) 20 column volumes gradient from 1.5 to 0.75 M ammonium sulfate in buffer B; (2) five column volumes gradient from 0.75 to 0 M ammonium sulfate in buffer B. 1 ml fractions were collected. The peak containing ABALDH activity was eluted from 1.12 to 0.99 M ammonium sulfate concentration. Active fractions were pooled and desalted by gel-filtration on the PD10 desalting column (Amersham Pharmacia Biotech, UK) equilibrated with buffer C (50 mM potassium phosphate, pH 7.4, 1 mM DTT and 10% glycerol). The purified enzyme was aliquoted and stored at −70 °C until used.

The protein concentrations of enzyme preparations were estimated following the method described by Bradford [18] with bovine serum albumin as the standard. Proteins were separated by SDS/PAGE [19] with 12% polyacrylamide resolving gel.

2.7. Determination of ABALDH molecular weight

The molecular weight of purified ABALDH was determined by gel-filtration using Superdex 200 HR 10/30 (Amersham Pharmacia Biotech, UK) column 0.1×31 cm according to Amersham Pharmacia Biotech manufacturer's recommendation. Isocratic elution was done with 50 mM potassium phosphate, pH 7.4. The column was calibrated with gel-filtration molecular weight (M_w) markers (Calbiochem, Pharmacia): ferritin (445 000 Da), aldolase (158 000 Da), egg albumin (45 000 Da) and myoglobin (17 000 Da). The calibration carve was determined as $\log(M_w) = 3.09 - 3.31 \times K_{av}$ by means of linear regression of the experimental points (SigmaPlot 8.0). The K_{av} for the individual proteins were calculated as $(V_R - V_O)/(V_C - V_O)$, where V_O – void volume of the column, V_R – retention (elution) volume of the protein, V_C – the geometric bed volume.

2.8. Mass spectrometry analysis

Mass spectrometry analysis of the protein samples was carried out in Proteomic Research Department, Institute of Biomedical Chemistry, Moscow, Russia. Treatment of gels, trypsinolysis, protein extraction and mass analysis by time-of-flight matrix-assisted laser desorption-ionization (MALDI-TOF) were carried out according to protocols described by Govorun et al [20]. Protein was identified by the set of its proteolytic peptide masses using Peptide Fingerprint option of Mascot software (Matrix Science, USA, http://www.matrixscience.com). The accuracy of MH^+ mass determination was 0.01% and possible modification of cysteine residues by acrylamide and methionine oxidation were taken into consideration. For searching procedure the complete genome *E. coli* K12 MG1655 database [21] of US National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov) was employed.

3. Results and discussion

Owing to its long history, the problem of identification of the gene encoding ABALDH in *E. coli* remained open till now. The gene encoding ABALDH was mapped only approximately between 28 and 32 min of *E. coli* chromosome by conjugation [11]. Searching through the *E. coli* K12 MG1655 genome [21] for the nearest homologues of the human ABALDH (P49189) revealed nine ORFs, having highest identity parameter: BetB (P17475, 51%), [1] YneI (P76149, 39%), YdcW (P77674, 36%), HpcC (P42269, 35%), AldA (P25553, 35%), AldB (P37685, 34%), GabD (P25526, 34%), FeaB (P80668, 34%) and AldH (P23883, 32%). Three of the genes, encoding YdcW, AldA and AldH, are localized between 28 and 32 min of the chromosome. Each of them was characterized as aldehyde dehydrogenases

[1] In brackets protein identification number in SWISS-PROT Database and identity parameter are shown.

Table 3
Kinetic parameters of *E. coli* YdcW protein

Substrate	V_{max} (μmol/min/mg)	K_m (μM)	k_{cat} (1/min)	k_{cat}/K_m (1/min/μM)
ABAL[a]	9.08 ± 0.42	41 ± 7	461 ± 17	11.25
Butyraldehyde[a]	0.36 ± 0.02	196 ± 40	18 ± 1	0.09
NAD^+[b]	6.67 ± 0.36	54 ± 10	339 ± 14	6.27
$NADP^+$[b]	2.51 ± 0.2	484 ± 72	127 ± 4	0.26

[a]0.5 mM NAD^+ was used as coenzyme.
[b]0.5 mM ABAL was used as substrate.

previously [22–24], but nothing was known about theirs affinity for ABAL.

We used classical approach comprising purification and identification of ABALDH protein(s) from the wild-type *E. coli* K12 strain. We purified ABALDH from MG1655 cells grown in LB-broth when relatively high specific activity (Table 1) and maximum cells growth rate were achieved. The procedure for ABALDH purification from soluble fraction of crude cells extract was developed using precipitation in ammonium sulfate solution, anion-exchange chromatography and hydrophobic interaction chromatography. The used procedure resulted in 353-fold ABALDH purification with 15% yield (Table 2). At every chromatographic stage of the purification process the single ABALDH activity peak was detected (data not shown). The homogeneity of the purified ABALDH was assessed by SDS/PAGE and a single band with molecular mass

A

Peptide		
№	Mr, Da	Sequence
1	739.38	FFAGAAR
2	843.53	LGAAVATLK
3	859.49	VITGGEKR
4	1037.57	GIYDTLVEK
5	1121.60	TVGDPLTGHPK
6	1253.69	AVEEAKATGHIK
7	1306.71	GKTVGDPLTGHPK
8	1563.73	AADAAFAEWGQTTPK
9	1660.77	DMSLYGLEDYTVVR
10	1676.77	DMSLYGLEDYTVVR Oxidation (M)
11	2013.98	APVIVFDDADIEAVVEGVR
12	2266.02	LSGYGKDMSLYGLEDYTVVR
13	2281.01	SGAPDDESTELGPLSSLAHLER
14	2442.13	NCGKPLHSAFNDEIPAIVDVFR
15	2496.23	MVSLTGSIATGEHIISHTASSIKR
16	2512.23	MVSLTGSIATGEHIISHTASSIKR Oxidation (M)
17	2867.35	THMELGGKAPVIVFDDADIEAVVEGVR
18	2883.35	THMELGGKAPVIVFDDADIEAVVEGVR Oxidation (M)
19	3998.84	EVFGPVVSVTPFDNEEQVVNWANDSQYGLASSVWTK

B

Fig. 2. Mass spectrometry analysis of purified ABALDH protein. (A) Mass spectrum of tryptic digested purified ABALDH and corresponding set of YdcW tryptic digested peptides. (B) Trypsin-digestion map of YdcW. Peptides matched with observed peptide mass spectra are marked by grey background. Proteolysis sites are depicted by vertical arrows.

of about 51 ± 3 kDa was obtained. The molecular weight of the native ABALDH was determined about 202 ± 29 kDa by gel-filtration, suggesting that the active form of the enzyme exists as a homotetramer.

Purified ABALDH protein was extracted from gel and digested by trypsin. Mass-analysis of the obtained peptide mixture was carried out by time-of-flight matrix-assisted laser desorption-ionization (MALDI-TOF). The set of 19 mass-peaks was observed (Fig. 2A). Searching employed *E. coli* MG1655 genome database [21] revealed that it is matched with YdcW (SWISS-PROT Database, P77674) trypsin-digestion peptide spectrum (Fig. 2B) exhibiting the high score parameter equal to 204 (score was determined as $-10 \times \log(P)$, where P is the probability that the observed match is a random event). Thus purified protein was identified as YdcW. This result was expected, as YdcW has the higher identity with human ABALDH and the corresponding gene is localized on 32.63 min of the chromosome.

Previously, His-tag fused YdcW enzyme was purified, crystallized and characterized as a medium chain aldehyde dehydrogenase having tetrameric structure [22]. We determined the kinetic parameters of the native YdcW in ABAL oxidation reaction (Table 3). The K_m values for ABAL, NAD^+ and $NADP^+$ were equal to 41 ± 7, 54 ± 10 and 484 ± 72 μM, respectively. They are comparable to those of ABALDH previously purified from *E. coli* K12 mutant [14]. In accordance with our data the catalytic efficiency (k_{cat}/K_m) of YdcW for NAD^+ was over one order of magnitude higher then that one for $NADP^+$ (Table 3), confirming the YdcW has much better affinity for NAD^+ than for $NADP^+$ [22]. The K_m parameter for ABAL of YdcW is appreciably lower than that for any one of 14 medium chain aldehydes examined by Gruez et al. [22]. We examined the catalytic efficiency of the native YdcW for butyraldehyde, which is one of the most effective substrates previously assayed [22]. The k_{cat}/K_m ratio for butyraldehyde (Table 3) is more then two order of magnitude lower as that one for ABAL, supposing that catalytic efficiency for other aldehydes examined by Gruez et al. [22] is appreciably lower too. Summarizing, we can suggested that ABAL is the native substrate of YdcW and the primary biochemical function of YdcW is oxidation of ABAL to GABA.

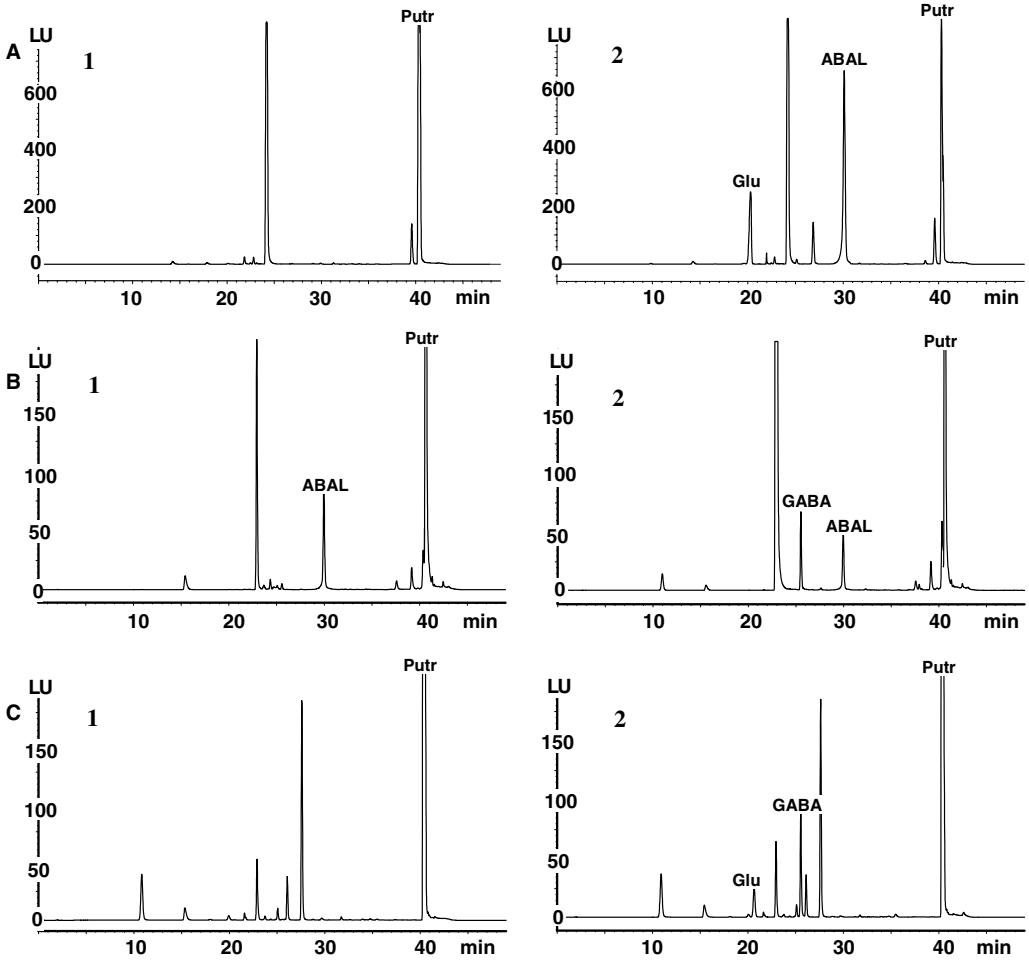

Fig. 3. HPLC-monitoring of the reactions components. (A) Enzymatic ABAL and L-glutamic acid (Glu) formation: (1) starting reaction mixture containing putrescine (Putr) and α-ketoglutarate; (2) reaction mixture after incubation with YgjG transaminase. (B) Enzymatic oxidation of ABAL to GABA: (1) starting reaction mixture containing ABAL and NAD^+; (2) reaction mixture after incubation with YdcW dehydrogenase. (C) Enzymatic interconversion of putrescine to GABA: (1) Starting reaction mixture containing putrescine, α-ketoglutarate and NAD^+; (2) reaction mixture after incubation with YgjG and YdcW.

Study of ABALDH induction in MG1655 (Table 1) revealed that it is under the same dual control (catabolic repression and nitrogen limitation) as it was described for the previously investigated *E. coli* K12 mutants which can grow in putrescine as the sole carbon and nitrogen source [25]. The inactivation of *ydcW* gene in MG1655 resulted in disappearance of ABALDH activity in crude cell extracts of MG1655 Δ*ydc*W::kan strain cultivated in all examined cultural media (see Table 1). In addition accumulation of ABAL (0.76 mM) was observed in the culture medium of MG1655 Δ*ydc*W::kan strain under nitrogen starvation conditions. ABAL was not detected in cultural medium of MG1655 cultivated at the same conditions. According to these data we suggested that YdcW is the unique ABALDH in the wild-type *E. coli* K12.

In order to investigate the coupling action of putrescine transaminase and ABAL dehydrogenase, the time-depending interconversion of reaction's components in vitro was monitored by HPLC-analysis. At first, we examined ABAL and L-glutamate formation in PAT reaction with purified YgjG ([13], Fig. 3A) and GABA formation from chemically obtained ABAL in NAD^+-dependent ABALDH reaction with purified YdcW enzyme (Fig. 3B). Then it was showed that incubation of putrescine and α-ketoglutarate at the presence of NAD^+, PLP and purified YgjG and YdcW enzymes resulted in accumulation of GABA (Fig. 3C). Thus in vitro coupling action of the purified *E. coli* K12 YgjG and YdcW enzymes results in conversion of putrescine into GABA through ABAL.

Summarizing, wild-type *E. coli* K12 possess an active PAT [13] and unique ABALDH enzyme, suggesting that the joint action of these enzymes is important to maintenance of the intracellular putrescine homeostasis in *E. coli* K12.

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