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RESEARCH ARTICLE

Purification and immobilization of L-arginase from thermotolerant *Penicillium chrysogenum* KJ185377.1; with unique kinetic properties as thermostable anticancer enzyme

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Abstract L-Arginase, hydrolyzing L-arginine to L-ornithine and urea, is a powerful anticancer, L-arginine-depleting agent, against argininosuccinate synthase expressing tumors. Otherwise, the higher antigenicity and lower thermal stability of this enzyme was the main biochemical hurdles. Since, the intrinsic thermal stability of enzymes follow the physiological temperature of their producer, thus, characterization of L-arginase from thermotolerant *Penicillium chrysogenum* was the objective of this study. L-Arginase (Arg) was purified to its homogeneity from *P. chrysogenum* by 10.1-fold, with 37.0 kDa under denaturing PAGE, optimum reaction at 50 °C, pH stability (6.8–7.9), with highest molar ratio of constitutional arginine, glutamic acid, lysine and aspartic acid. The purified enzyme was PEGylated and immobilized on chitosan, with 41.9 and 22.1 % yield of immobilization. At 40 °C, the $T_{1/2}$ value of free-Arg, PEG-Arg and Chit-Arg was 10.4, 15.6, 20.5 h, respectively. The free-Arg and Chit-Arg have a higher affinity to L-arginine (K_m 4.8 mM), while, PEG-Arg affinity was decreased by about 3 fold (K_m 15.2 mM). The inhibitory constants to the free and PEG-Arg were relatively similar towards HA and PPG. The IC_{50} for the free enzyme against HEPG-2 and A549 tumor cells was 0.136 and 0.165 U/ml, comparing to 0.232 and 0.496 U/ml for PEG-Arg, respectively. The in vivo $T_{1/2}$ to the free Arg and PEG-Arg was 16.4 and 20.4 h, respectively as holoenzyme. The residual L-arginine level upon using free Arg was 156.9 and 144.5 μM, after 6 and 8 h, respectively, regarding to initials at 253.6 μM, while for Peg-Arg the level of L-arginine was nil till 7 h of initial dosing. The titer of IgG was induced by 10–15 % in response to free-Arg after 28 days comparing to IgG titer for PEG-Arg.

Keywords Thermotolerant *Penicillium chrysogenum* · L-Arginase · Thermal stability · Anticancer · Antigenicity

Introduction

L-Arginase (L-arginine amidinohydrolase, EC 3.5.3.1), is a metalloenzyme catalyzes the hydrolysis of L-arginine to L-ornithine and urea (Borkovich and Weiss 1987). L-Arginase is a pivotal enzyme in ammonia detoxification and generation of urea in liver and kidney, source of L-ornithine to proline and glutamate synthesis (Jenkinson et al. 1996). In mammalian tissues, there is L-arginase I and II which their coding genes are located in chromosome 6 and 14, and expressed in liver and kidney, respectively (Wu and Morris 1998). L-Arginase II is mainly involved in synthesis of L-ornithine as precursors of polyamines through ornithine decarboxylase, spermidine synthase and spermine synthase (Jenkinson et al. 1996). Spermine is the predominant polyamine in human tumors like breast carcinoma, increased by three fold than in healthy tissues (Singh et al. 2000). Biochemically, L-arginase mainly regulates the growth and proliferation of tumor cells by polyamine synthesis.

Recently, L-arginase was documented as a powerful anticancer drug by mediating L-arginine depletion (Cavanaugh and Nicolson 2000; Philip et al. 2003), especially against hepatocellular carcinoma (Cheng et al. 2007) and malignant

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melanoma (Feun and Savaraj 2006), that express argininosuccinate synthase (ASS), in contrary to action of L-arginine deiminase (Munder 2009). The anticancer activity of this enzyme was frequently ascribed to its dual activity as nitric oxide synthase, metabolizing arginine to citrulline and nitric oxide (NO) via N^{ω}-hydroxy-L-arginine (NOHA) intermediate (Edwards et al. 1996; Jenkins et al. 1995). The significant tumor apoptosis by the higher NO levels was reported (Farias-Eisner et al. 1994). Dietary L-arginine was observed to be enhances the tumor cell proliferation, in contrary to strongly inhibition of metastatic growth by L-arginine restriction (Cheng et al. 2007; Gonzalez and Byus 1991).

L-Arginase was characterized from *Bacillus brevis* (Kanda et al. 1997), *B. anthracis* (Viator et al. 2008), *Helicobacter pylori* (Zhang et al. 2011), and mammalian tissues (Munder, 2009). However, few studies were documented to the enzyme from fungi, except *Neurospora crassa* (Borkovich and Weiss 1987) and *Aspergillus nidulans* (Borsuk et al. 1999). Regarding to the relative higher antigenicity, rapid proteolysis and lower thermal stability of currently used arginase, PEGylation trials to improve its therapeutic potency were conducted (Cheng et al. 2007). The higher antigenicity might be due to the surface antigenic epitopes, for the stereo structural orientation of enzyme, since the clinically tried enzyme was of bacterial origin. It was though, that enzyme from eukaryotic origin should be more compatible therapeutically in humans, than bacterial ones. Thus, antigenicity and thermal instability were the main reported biochemically defects for clinical trials of this enzyme. Thus, we tried, by this work, to resolve the two problems, by purifying L-arginase from thermophilic fungus, to overcome both antigenicity and thermal labiality. Since, the intrinsic thermal stability of enzymes usually follows the physiological temperature of the producer microbes (Haki and Rakshit 2003; Maheshwari et al. 2000). There is no reports describing L-arginase from thermophilic microbes, thus, purification of this enzyme from thermophilic fungi was the challenge.

The objective of this study was to characterize L-arginase from thermotolerant *Penicillium chrysogenum*. As well as, to maximally improve the chemical properties of this enzyme by conjugation on PEG moieties and covalent immobilization on activated chitosan. Also, to evaluate the pharmacokinetic properties of these enzymes in vivo, in addition to their anticancer potency, against various tumor cell, in vitro.

Materials and methods

Materials

Methoxypolyethyleneglycol 5,000 propionic acid *N*-succinimidyl ester (PEG), chitosan, propargylglycine, 6-diazo-5-oxo-norleucine (DON), DEAE-Sepharose, ninhydrin reagent and diacetylmonoxime were purchased from Sigma-Aldrich Co. (Spruce St. Louis Mo, USA). All of the other chemical were of analytical grade.

Thermophilic fungal isolates and culture conditions for L-arginase production

Penicillium chrysogenum KJ185377.1 was selected as the potent isolate producing L-arginase (Data not shown), grown at 45 °C, on modified Dox's medium (Rapper and Fennell 1965) of L-arginine as sole nitrogen and carbon source. *P. chrysogenum* KJ185377.1 was identified based on morphological and molecular analysis (rDNA sequence). Briefly, the medium contains; 0.5 % L-arginine, 0.2 % KH_2PO_4, 0.05 % $MgSO_4$. $7H_2O$, 0.05 % KCl, dissolved in tap water, the pH was adjusted to 7.0. Five ml of the spore suspension (5 days old fungal slab cultures, 10^{7-8} spore/ml) was inoculated to 100 ml medium/250 ml Erlenmeyer conical flasks. The cultures were incubated at 45 °C for 6 days under shaking conditions (120 rpm), filtered and centrifuged. The activity of L-arginase and its concentration was measured.

L-Arginase assay

L-Arginase activity was determined based on the released ornithine by ninhydrin assay (Zhang et al. 2011). Briefly, the reaction contains 500 µl L-arginine (100 mM) in potassium phosphate buffer (pH 7.0) of 1 mM $MnCl_2$, and 500 µl of the enzyme preparation. Blanks of enzyme and substrate were conducted. The reaction mixture was incubated for 10 min at 40 °C, then 100 µl of 10 % TCA was added, followed by 25 µl of ninhydrin reagent. The mixture was boiled for 5 min, centrifuged and the developed color was measured at 575 nm. One unit of L-arginase was expressed by the amount of enzyme releasing 1 µM of L-ornithine under standard assay, based on authentic L-ornithine.

The enzyme protein content was determined by Folin's reagent (Lowry et al. 1951).

Purification of L-arginase

L-Arginase was purified from the submerged cultures of *P. chrysogenum* incubated at 45 °C by salting out, ion-exchange and gel-filtration chromatography according to our previous studies (El-Sayed, 2011; El-Sayed et al. 2012; El-Sayed et al. 2013; El-Sayed et al. 2014), three hundred grams of the fungal pellets were collected, washed and used as enzyme source.

The molecular homogeneity and subunit structure of the purified enzyme was checked by the denaturing and non-denaturing-PAGE (Laemmli 1970).

The amino acid constitution of the purified enzyme was determined by Amino Acid Analyzer (Moore et al. 1958).

Immobilization of the purified L-arginase

The purified L-arginase was homo-functionally PEGylated on 5 kDa PEG propionic acid *N*-succinimidyl ester by molar ratio 1/50 (CGL: PEG), in potassium phosphate buffer pH (7.5) and purified by size exclusion chromatography (Sun et al. 2003).

The purified L-arginase was covalently immobilized on chitosan, activated by glutarldehyde (El-Sayed and Shindia 2011). The immobilization yield was expressed by the ratio of specific activity of immobilized enzyme to free one.

The modification of surface primary amino groups, ε-amino groups of lysine and surface thiols were determined (El-Sayed and Shindia 2011).

Biochemical, catalytic properties and resistance to proteolysis of the enzymes

The biochemical properties as optimum pH, pH, thermal stability and inhibitors were assessed (El-Sayed 2011; El-Sayed and Shindia 2011). Also, the resistance of free and immobilized L-arginase to in vitro proteolysis by trypsin and proteinase K was evaluated based on their colorimetric activity and molecular homogeneity by native-PAGE.

Pharmacokinetic and antigenic properties of the free and PEGylated L-arginase

The pharmacokinetics properties of the free and PEGylated enzyme was assessed using White New Zealand Rabbits (five rabbits per group, each one was 1.5 ± 0.3 kg), i.v. injected by single doses of enzyme (Yang et al. 2004a). Positive and negative controls were used. The blood was collected intervally, the biological half-life, and residual plasma L-arginine was determined by HPLC analysis (Yang et al. 2004b). Various biochemical and hematological parameters was evaluated in vivo to assess the cytotoxicity of the enzymes.

The antigenic properties was assessed by injection of the rabbits by three successive doses of free and PEGylated L-arginase, along 20 days of initial injection. The amount of antienzymes immunoglobulins (IgG) was determined by Radial Immuno-Diffusion Kit.

Anticancer activity of purified L-arginase

The cytotoxic activity of free and modified enzyme against human lung carcinoma (A549) and hepatic cellular carcinoma (HEPG-2) was evaluated by MTT assay (Hansen and Hansen 1989). This assay based on activity of mitochondrial dehydrogenase of living cells to convert the yellow MTT tetrazolium rings to black impermeable formazan crystals, measured at 570 nm (Hansen and Hansen 1989).

Statistical analysis

All the experiments were conducted in triplicates, and the presented data are mean ±SD, calculated using Excel 2007.

Results

Purification, molecular structure and amino acid contents

L-Arginase was purified to its electrophoretic homogeneity from *P. chrysogenum* by about 10.02-fold with 0.72 % yield (Table 1), by salting out, DEAE-Sepharose and Sephadex G_{100} columns. The most active fractions from each column were selected based on their colorimetric activity and molecular homogeneity by SDS-PAGE. The selected fractions were gathered, concentrated by polyethyleneglycol to minimum volume (10 ml).

The molecular subunits structure of the purified L-arginase from the cultures of *P. chrysogenum* was determined by denaturing PAGE (Fig. 1), with 37.0 kDa. The amino acids content of purified L-arginase from thermotolerant *P. chrysogenum* were measured (Table 2). The enzyme contains a relatively higher polar positive amino acids (L-arginine 13.7 %, L-lysine 8.1 %) than negative ones (glutamic 11.13 % and aspartic acid 7.89 %), assuming the relatively basic identity of this enzyme. While, a fairly amounts of neutral amino acids was detected as leucine (10.1 %), alanine (9.2 %), methionine (6.9 %), phenylalanine (5.8 %) and

Table 1 Overall purification profile of arginase from *Penicillium chrysogenum*

Step of purification	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield
Crude enzyme	1,008	412	2.44	1	100
Ammonium sulfate (30–70 %)	179.69	28.5	6.30	2.58	17.83
Ion-exchange chromatography	19.59	1.937	10.11	4.14	1.94
Gel-filtration	7.26	0.297	24.45	10.02	0.72

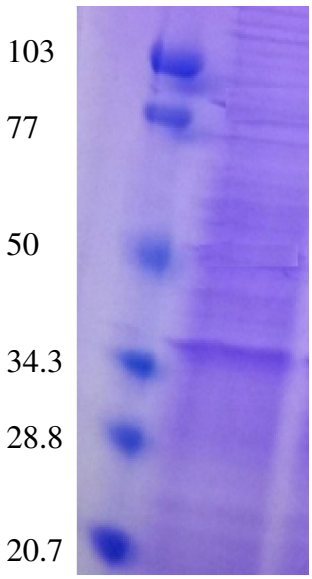

Fig. 1 SDS-PAGE of the purified L-arginase from *P. chrysogenum* by fractional precipitation by salting out, ion-exchange and gel-filtration chromatography, as described in "Materials and methods" section

valine (2.6 %). The aromatic amino acids were represented by about 12 % (histidine, phenylalanine, tyrosine), however, the polar basic positive amid amino acids as glutamine and asparagine were not detected during the amino acid analysis.

Immobilization of *P. chrysogenum* L-arginase

The purified enzyme was PEGylated and immobilized on chitosan according to our previous protocols (El-Sayed and shindia 2011). The immobilization yield was 41.9 and 22.1 % for PEG-Arginase and Chit-Arginase, respectively, based on their colorimetric activity. The specific activity of PEG-arginase and Chit-arginase was 10.26 U/mg and 5.39 U/mg, respectively, comparing to free enzyme (24.4 U/mg). So, the initial activity of PEG, and Chit-arginase was decreased by about 59 and 78 %, revealing the little interaction of PEG moieties with surface functional sites, comparing to nonspecific high interaction by chitosan. The degree of modifications of surface lysine ε-amino group by immobilization was evaluated using Fluorescamine assay (El-Sayed et al. 2014). From the fluorescence emitted spectra, the surface ε-amino groups of PEG-arginase was about 82.1 % to free enzyme, ensuring occupation of 18 % of surface lysine residues by interaction with PEG (Data not shown)..

Table 2 Amino acid constitution of the purified L-arginase from *P. chrysogenum*

Amino acid	Conc. (μg/ml)	Mole (%)
Aspartic	87.09	7.89
Theronine	38.31	3.47
Serine	64.20	5.81
Glutamic acid	122.85	11.13
Glycine	34.37	3.11
Alanine	102.35	9.27
Valine	28.82	2.61
Methionine	77.22	6.99
Isoleucine	35.63	3.22
Leucine	110.67	10.02
Tyrosine	5.98	0.54
Phenylalanine	64.10	5.80
Histidine	72.22	6.54
Lysine	88.56	8.02
Arginine	151.80	13.75
Proline	19.33	1.75

Biochemical properties on free and immobilized L-arginase

The thermal stability of the free and immobilized L-arginase was studied at 40, 50, 60, 70 °C, under standard assay. From the thermal kinetic parameters (Table 3), at 40 °C, the $T_{1/2}$ value to free-Arg, PEG-Arg and Chit-Arg was 10.4, 15.6, 20.5 h, with thermal inactivation rate (Kr) 0.006, 0.004, 0.003 min, respectively. Also, the thermal stabilizing factor (SF) was increased by about 1.55 and 1.8-fold at 60 °C, and by 1.25 and 1.7-fold at 70 °C, respectively, the half-life temperature (Tm) was assessed to be 62.44, 86.0 and 84.1 °C, to free-Arg, PEG-Arg and Chit-Arg, respectively.

Table 3 Thermal kinetic paramters of free arginase, PEG-arginase and Chit-arginase

Enzyme	$T_{1/2}$	Kr (min) $\times 10^{-3}$	SF (%)	Tm (°C)
Free arginase				
40	10.41	6.3	100	62.4
50	6.5	7.7	100	
60	5.78	9.51	100	
70	5.1	9.1	100	
PEG-arginase				
40	15.6	4.1	150.7	86.0
50	9.64	6.6	147.9	
60	8.9	7.6	154.8	
70	9.0	7.5	125.8	
Chit-arginase				
40	20.5	2.7	197.2	84.1
50	11.3	6.0	172.5	
60	10.7	5.5	185	
70	12.5	4.5	175	

Table 4 Substrate specificity of the free arginase, PEG-arginase, and Chit-arginase, based on the released byproducts

Amino acid	Ammonia releases (%)			Ornithine releases (%)			Keto acid releases (%)		
	Free-Arg	PEG-Arg	Chit-Arg	Free-Arg	PEG-Arg	Chit-Arg	Free-Arg	PEG-Arg	Chit-Arg
Arginine	–	–	–	100	100	100	100	100	100
Cystine	2	–	–	–	–	–	2.9	–	8
Lysine	120	130	96	–	–	–	–	–	55
Asparagine	167	168	–	12	–	–	–	–	–
Tyrosine	–	–	–	–	–	–	–	–	–
Cysteine	–	–	–	41	17	–	70.5	100	0
Alanine	150	190	–	–	–	–	–	–	100
Phenylalanine	145	128	–	44	10	100	–	–	–
Glycine	79	174	–	33	100	21	32.3	100	120
Methionine	–	–	–	–	–	–	–	–	–
Glutathione (GSH)	–	–	–	–	–	–	–	–	–

The substrate specificity of the free and modified L-arginase was assessed towards various amino acids, under the standard assay. The activity was determined based on released keto acid by Ninhydrin assay (Zhang et al. 2011), Diacetylmonoxime assay (Liu et al. 1995) and released ammonia by Nessler's reagent. From the data (Table 4), regarding to the maximum affinity for L-arginine, the free and immobilize L-arginase display a higher affinity to phenyl-alanine and glycine, with no detectable activity towards cysteine, lysine, tyrosine, asparagine and methionine based on keto acid releases as ornithine and citrulline. However, based on released ammonia, the enzyme displayed a fairly activity to L-asparagine and L-alanine. Thus, the L-arginine and phenylalanine was used for further enzymatic kinetic studies.

The kinetic parameters of the free and immobilized enzyme towards L-arginine and phenylalanine was determined using various concentrations of each one (10–50 mM), under standard assay. From the data (Table 5), the free Arg and Chit-Arg has the same affinity (K_m) (4.8 mM) for L-arginine, while, the affinity of PEG-Arg was decreased by about three fold (K_m 15.2 mM). As well as, the maximum velocity (V_{max}) towards L-arginine as substrate was observed to free enzyme (21.7 U/mg/min), followed by Chit-Arg (18.86 U/mg/min) and PEG-Arg (14.08 U/mg/min). However, the affinity of the free and immobilized enzyme to phenylalanine was decreased by 4.5-fold comparing to L-arginine, under standard assay.

Kinetics of L-arginase inhibition by suicide inhibitors

The kinetics of inhibition of free and immobilized enzyme was assessed in presence of iodoacetamide (IA), hydroxylamine (HA) and propargylglycine (PPG) (10–50 µM). From the obtained data (Table 6), for IA, the half-inhibitory concentration (IC_{50}) was 61.8, 65.8 and 36.2 µM to free-Arg, PEG-Arg and Chit-Arg, respectively. However, the free enzyme displayed a degree of resistance to HA and PPG, than immobilized one. The IC_{50} values for HA and PPG was 80.8 and 85.7 µM to the free enzyme and 65.6 and 62.6 µM for PEGylated one, respectively. The inhibitory constant (Ki) was calculated as proposed by Cheng and Prussoff (Cheng and Prusoff 1973), practically, the Ki values as non-competitive inhibition was higher than free and immobilized enzyme for the three inhibitors. The highest measured Ki values was 61.8, 80.8 and 85.7 µM, for free enzyme and 65.8 µM, 65.6 and 62.6 µM, for the PEGylated one, for IA, HA and PPG, respectively.

Proteolytic digestion of free and PEGylated L-arginase in vitro

The responses of free and PEGylated L-arginase to proteolytic digestion was assessed by incubation with proteinase K for 30 min at 35 °C, then measuring their residual activity by the standard assay. From the data (Fig. 2), the

Table 5 Kinetics of substrates specificity of free and immobilized arginase towards arginine and phenylalanine

Substrate	Free-arginase			PEG-arginase			Chit-arginase		
	K_m (mM)	V_{max} (U/mg/min)	K_{cat} (S^{-1})	K_m (mM)	V_{max} (U/mg/min)	K_{cat} (S^{-1})	K_m (mM)	V_{max} (U/mg/min)	K_{cat} (S^{-1})
Arginine	4.8	21.7	0.27	15.2	14.08	0.18	4.47	18.86	0.25
Phenylalanine	22.1	19.5	0.25	32.8	72.99	0.94	57.5	35.7	0.46

Table 6 Kinetic Inhibition of free and immobilized arginase by site specific/amino acid analogues

Enzyme	Iodoacetamide			Hydroxylamine			Propargylglycine		
	IC_{50} (mM)	Ki (μM)		IC_{50} (mM)	Ki (μM)		IC_{50} (mM)	Ki (μM)	
		Comp	Noncomp		Comp	Noncomp		Comp	Noncomp
Free-arginase	61.8	0.006	61.8	80.8	0.007	80.8	85.7	0.008	85.7
PEG-arginase	65.8	0.02	65.8	65.6	0.019	65.63	62.6	0.019	62.6
Chit-arginase	36.2	0.01	36.2	44.2	0.003	44.2	30.5	0.002	30.5

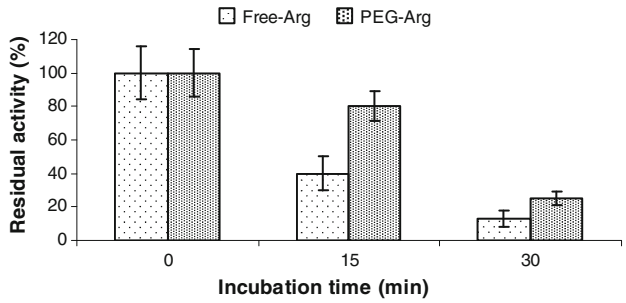

Fig. 2 Residual activity of free and PEG-Arginase upon digestion by proteinase K for 15 and 30 min, comparing to control (zero time). The proteinase digested enzymes were significantly different from the control $p < 0.05$

free enzyme retains about 51.1 and 15.3 %, after 15, and 30 min, while PEG-Arg retains 76.8 and 24 % of their initial activities, respectively. Thus, PEGylation could be masking the proteolytic recognition sites of proteinase K on the surface of L-arginase.

Antitumor efficiency of free and immobilized L-arginase

The anticancer efficiency of the free and PEG-Arg was assessed against hepatic cellular carcinoma and human lung carcinoma. From the cytotoxic activity (Table 7), the IC_{50} for the free enzyme against HEPG-2 and A549 tumor cells, was 0.11 and 0.18 U/ml, comparing to 0.24 and 0.496 U/ml for PEG-Arg, respectively. Thus, the anticancer efficacy of the PEG-Arg was reduced by two and three fold for HEPG-2 and A549, respectively, comparing to free enzyme.

Pharmacokinetics properties and L-arginine concentration in response to free Arg and PEG-Arg

The pharmacokinetic properties of free and PEG-L-arginase were determined after single dosing of New Zealand rabbits. The in vivo biological $T_{1/2}$ to free and PEG-Arg was 16.4 and 20.4 h, respectively, as holo-enzymes, without Mn^{+} cofactor during the assay. However, using Mn^{+} during the assay, the half-life time to free and PEGylated L-arginase was increased to 35 h. So, the activity L-arginase

Table 7 Cytotoxic activity of free and PEG-Arginase against hepatocellular carcinoma and human lung cancer

Enzyme	Hepatocellular carcinoma HEPG-2 (IC_{50})	Human lung carcinoma A549 (IC_{50})
Free-Arginase	0.11 ± 0.07	0.18 ± 0.09
PEG-Arginase	0.24 ± 0.08	0.28 ± 0.08

forms was increased by 1.8-to 2-fold upon addition of Mn^{+} as cofactor, during the assay. Thus, Mn^{+} (0.1 mM) was externally infused to rabbits after about 30 h of initial enzyme dosing, to assess the metal dependent activity of L-arginase in vivo. From the obtained results, the activity of both forms of L-arginase was increased maximally by 85 % to initial activity after 6 h, followed by sequential decreasing on their activities.

The in vivo concentration of blood L-arginine was determined intervally parallel to enzyme kinetics by HPLC, for the rabbits injected with free and PEGylated L-arginase. From the in vivo profile of L-arginine (Fig. 3), the residual concentration of L-arginine upon using free enzyme was 156.9 and 144.5 μM, after 6 and 8 h, respectively, regarding to negative controls at 253.6 μM. However, the concentration of L-arginine was 30 μM, after 2, 4, 6 h of initial dosing by PEG-Arg, and resumed to 107.6 μM, after 8 h. Thus, the concentration of L-arginine was strongly decreased till 7 h of initial dosing by PEG-Arginase comparing to free enzyme, assuming the protective action of PEG on enzyme catalytic structure.

Biochemical and hematological parameters of rabbits injected by free and PEG-Arg

The biochemical and hematological parameters of the rabbits in response to free and PEGylated enzyme was determined intervally. From the profile of blood biochemical and hematological analyses (Supplementary Figures), the free and PEG-Arg have no detectable negative effect on various measured blood parameters except, platelet concentration that decreased by about 35–40 % after 10–15 h, then

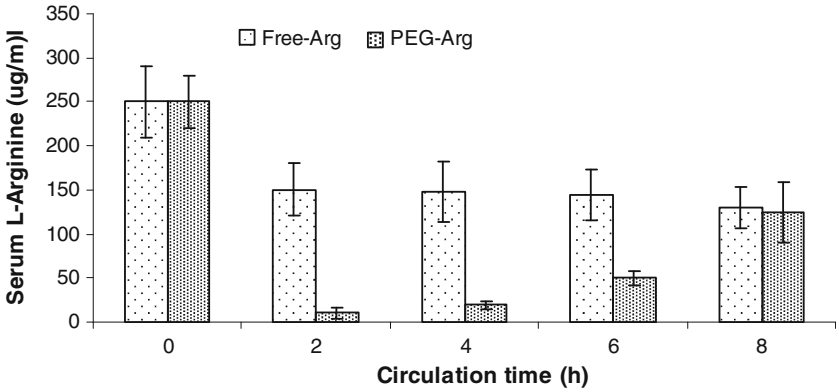

Fig. 3 Concentration of serum arginine upon single dosing of free and PEG-arginase in vivo in New Zealand rabbits. The concentrations of serum L-arginine was significantly different from controls $p < 0.01$

resumed again to its normal concentrations. Otherwise, the concentration of urea was increased by about 15–20 %, after 6–10 h of initial injection of free and PEGylated L-arginase.

Antigenic properties of free and PEG-Arg

The titer of IgG immunoglobulins in response to free and PEG-Arg were quantified by the RID assay Kit ("Materials and methods" section). As shown from the results (Fig. 4), the free Arg has a slightly inducing effect on IgG titer by about 10–15 % after 28 days of injection comparing to the unchanged titer of IgG for PEG-Arg. Interestingly, the titer of IgG in response to PEG-Arg was relatively unchanged along the experimented 45 days from initial injection, unlike to free Arg.

Discussion

L-Arginase was documented as promising anticancer agent towards various tumors especially expressing ASS and ASL (Cheng et al. 2007; Philip et al. 2003), unlike to action of L-arginine deiminase (Munder 2009). Recently, the main cited biochemical therapeutic hurdles for L-arginase were the higher antigenicity, rapid plasma clearance, high proteolysis and lower thermal stability (Cheng et al. 2007). Thus, characterization of thermostable L-arginase from thermotolerant fungi was the main objective of the current study, assuming its favored properties. Therapeutic enzymes from eukaryotes are more compatible biochemically with human immune system (El-Sayed 2010). Particularly, thermophilic microbes have more intrinsic thermal stability enzyme system (Haki and Rakshit 2003; Maheshwari et al. 2000).

L-Arginase was purified from the thermotolerant *P. chrysogenum* by about 10.02-fold with 0.72 % yield. However, the enzyme was purified from mesophilic fungi, optimally grow at 30 °C, as *N. crassa* (Borkovich and Weiss 1987), *S. cerevisiae* (Penninckx et al. 1974) and *A. nidulans* (Dzikowska et al. 1994).

The molecular weight of purified *P. chrysogenum* L-arginase was 37.0 kDa under denaturing PAGE, a single proteinous units reveals the reliability of the designed purification protocol. Consistently, L-arginase from *A. nidulans*, *S. cerevisiae* (Dzikowska et al. 1994) and *N. crassa* (Borkovich and Weiss 1987) has the same subunit structure. However, L-arginase from *B. licheniformis* (Simon and Stalon 1976), *Iris hollandica* (Boutin 1982), and soybean (Kang and Cho 1990) has 33, 36.5, 35 and 60 kDa, respectively.

The enzyme contains a relative higher polar positive amino acids (L-arginine and L-lysine) then negative ones (glutamic and aspartic acid), assuming its basic identity, comparable to its optimum activity around pH 6.0–7.5. The enzyme contain higher ratio of polar amino acids to non-polar ones, as signs of its thermal stability (Vieille and Zeikus 2001), similar pattern of *Mycobacterium arthriditis* ADI (Weickmann et al. 1978) was reported. Practically, the dominance of L-arginine is a relevant sign of thermal stability, since its δ-guanido amino groups has a very low reactivity(Argos et al. 1979). Otherwise, the undetectable amid amino acids ensures the enzyme thermal stability, since these residues are highly thermo labile. The constitutional amino acid of the purified L-arginase approves its thermal stability (Vieille and Zeikus 2001), since rapid thermal inactivation was one of the main obstacles for practical uses. Further crystallographic and amino sequence analysis required to elucidate this hypothesis. The amino acid structure was strongly correlated with the catalytic thermal stability at higher temperatures.

The purified thermotolerant *P. chrysogenum* L-arginase was PEGylated and immobilized on chitosan, to explore its maximum catalytic stability, however, the PEG-Arg and Chit-Arg, retains about 50 and 25 %, comparing to native enzyme. The higher decreases on Chit-Arg than PEG-Arg activities could be attributed to the non-specific interaction of aldehyde group of glutaraldehyde linker with various amino groups of surface active sites, or denaturation of enzyme, comparing to precise reaction of PEG with surface ε-amines (El-Sayed et al. 2014; Ensor et al. 2002).

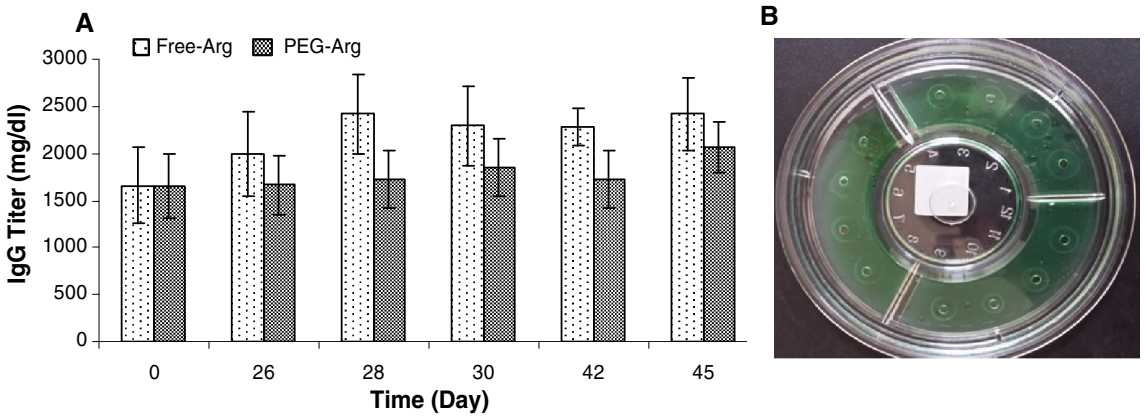

Fig. 4 Antigenic properties of free and PEG-Arg in New Zealand Rabbits. The titer of IgG in response to free-Arg and PEG-Arg (A) and precipitation zones on RID assay Kit (B), was determined. The titer of IgG for both enzymes was $p < 0.005$

The optimum pH stability of native, PEG and Chit-Arg around 6.0–7.5, seems to be favored criterion, since the pH of blood plasma around 7.0–7.5. L-Arginase from *Rhodobacter capsulatus* (Moreno-Vivian et al. 1992) has pH stability at 8.5–9.0. The slight alkaline stability of the enzyme seems to be comparable to its relative basic amino acids constitution.

The enzyme displayed a plausible thermal stability, at 40 °C, the $T_{1/2}$ value to the free-Arg, PEG-Arg and Chit-Arg was 10.4, 15.6, 20.5 h, with thermal inactivation rate (Kr) 0.0063, 0.0041, 0.0027 min, respectively, in vitro. The stability of free arginase was increased by 1.5 and 1.8-fold for Peg-Arg and Chit-Arg, ensuring the protective effect of immobilization on enzyme tertiary structure against thermal denaturation. However, the $T_{1/2}$ of L-arginase from *B. caldovelox* was 4.5 min at 60 °C (Patchett et al. 1991).

The free and immobilize arginase displayed a higher affinity for L-arginine, followed by phenylalanine, glycine with no detectable activity on cysteine, lysine, tyrosine, asparagine and methionine based on keto acid release. Kinetically, the affinity of free and immobilized enzyme to phenylalanine was decreased by 4.5-fold than L-arginine. Consistently, the affinity of *S. cerevisiae* arginase (Green et al. 1990) to arginine was 15.5 mM. The current enzyme displayed a higher affinity to L-arginine than *N. crassa* (K_m 131 mM) (Borkovich and Weiss 1987) and *R. capsulatus* (K_m 16 mM) (Moreno-Vivian et al. 1992) arginase, unlike the higher affinity of *B. caldovelox* arginase (3.5 mM) (Patchett et al. 1991). Interestingly, the K_{cat} of arginase were dramatically increased for enzyme from eukaryotes than prokaryotic sources (http://www.brenda-enzymes).

The effect of surface amino acids analogues on the enzyme activities was assessed. The IC_{50} by IA was quite similar to free and PEG enzyme, revealing the non-implications of PEG moieties with enzyme surface thiols. Practically, surface amino acids embracing active sites, was frequently deduced by using of specific amino acid analogues (El-Sayed et al. 2014; Schmidt and Dringen 2009).

After 15 min of proteinase K digestion, the free and PEG-enzyme retain 51.1 and 76.8 %, respectively, endorsing the masking of proteolytic recognition sites on arginase surface. Consistently, shielding of the surface proteolytic recognition sites on the target enzyme by PEGylation was extensively reported (Kotzia et al. 2007; Yang et al. 2004a).

From the in vitro cytotoxicity, the free enzyme displays a higher activity against HEPG-2 (IC_{50} 0.136 U/ml) and A549 (IC_{50} 0.165 U/ml) tumors, by about two fold comparing to PEG-Arg. The strong inhibition of these tumors growth by action of arginase, ensure its arginine auxotrophic identity (Delage et al. 2010; Philip et al. 2003). Consistently, arginase displayed a powerful activity against ASS positive hepatocellular carcinoma and lymphoblastic leukemia (Hernandez et al. 2010; Kim et al. 2007), chinese hamster lung and L5178Y lymphoma cells (Currie et al. 1979).

The $T_{1/2}$ in vivo to the free (16.4 h) and PEG- arginase (20.4 h) was assessed as holo-enzymes, while their activities were increased by 1.8-to 2-fold by addition of Mn^{+} as cofactor. The residual arginine upon using free enzyme was decreased to its half-initial levels after 8 h, otherwise, the arginine was not detected from 2 to 6 h of initial dosing by PEG-Arg (Kelly et al. 2012). From the biochemical and hematological analyses, free and PEG-Arg have no detectable effect on various blood parameters except, platelet level that was slightly decreased after 10–15 h. The free enzyme has a slightly inducing effect on the titer of IgG after 28 days of injection comparing to the PEG-Arg.

In conclusion, thermostable L-arginase was purified from the thermotolerant *P. chrysogenum*, grown optimally at 45 °C. PEGylation and covalent immobilization of the

purified enzyme on chitosan, kinetic properties and biochemical characterization were studied. The proteolytic response to proteinase K and active site prediction by chemical specific inhibitors was assessed. The in vivo pharmacokinetic properties and in vitro cytotoxic effect of free and PEG-arginase against HEPG-2 and A549 were evaluated. Actually, this is the first study to explore arginase from thermotolerant fungi, with unique catalytic and pharmacokinetic properties regarding to currently clinically used enzyme.

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Conflict of interest No conflict of interest.

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