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Aspergillus nidulans thermostable arginine deiminase-Dextran conjugates with enhanced molecular stability, proteolytic resistance, pharmacokinetic properties and anticancer activity

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ABSTRACT

The potential anticancer activity of arginine deiminase (ADI) *via* deimination of L-arginine into citrulline has been extensively verified against various arginine-auxotrophic tumors, however, the higher antigenicity, structural instability and *in vivo* proteolysis are the major challenges that limit this enzyme from further clinical implementation. Since, this clinically applied enzyme was derived from *Mycobacterium* spp, thus, searching for ADI from eukaryotic microbes "especially thermophilic fungi" could have a novel biochemical, conformational and catalytic properties. *Aspergillus nidulans* ADI was purified with 5.3 folds, with molecular subunit structure 48 kDa and entire molecular mass 120 kDa, ensuring its homotrimeric identity. The peptide fingerprinting analysis revealing the domain Glu^{95}-Gly^{96}-Gly^{97} as the conserved active site of *A. nidulans* ADI, with higher proximity to *Mycobacterium* ADI clade IV. In an endeavor to fortify the structural stability and anticancer activity of *A. nidulans* ADI, the enzyme was chemically modified with dextran. The optimal activity of Dextran-ADI conjugates was determined at 0.08:20 M ratio of ADI: Dextran, with an overall increase to ADI molecular subunit mass to ~100 kDa. ADI was conjugated with dextran *via* the ε-amino groups interaction of surface lysine residues of ADI. The resistance of Dextran-ADI conjugate to proteolysis had been increased by 2.5 folds to proteinase K and trypsin, suggesting the shielding of > 50% of ADI surface proteolytic recognition sites. The native and Dextran-ADI conjugates have the same optimum reaction temperature (37 °C), reaction pH and pH stability (7.0–8.0) with dependency on K^+ ions as a cofactor. Dextran-ADI conjugates exhibited a higher thermal stability by ~ 2 folds for all the tested temperatures, ensuring the acquired structural and catalytic stability upon dextran conjugation. Dextran conjugation slightly protect the reactive amino and thiols groups of surface amino acids of ADI from amino acids suicide inhibitors. The affinity of ADI was increased by 5.3 folds to free L-arginine with a dramatic reduction in citrullination of peptidylarginine residues upon dextran conjugation. The anticancer activity of ADI to breast (MCF-7), liver (HepG-2) and colon (HCT8, HT29, DLD1 and LS174 T) cancer cell lines was increased by 1.7 folds with dextran conjugation *in vitro*. Pharmacokinetically, the half-life time of ADI was increased by 1.7 folds upon dextran conjugation, *in vivo*. From the biochemical and hematological parameters, ADIs had no signs of toxicity to the experimental animals. In addition to the dramatic reduction of L-arginine in serum, citrulline level was increased by 2.5 folds upon dextran conjugation of ADI. This is first report exploring thermostable ADI from thermophilic *A. nidulans* with robust structural stability, catalytic efficiency and proteolytic resistance.

1. Introduction

Auxotrophic metabolic identity of tumor cells for plasma L-arginine for their rapid cellular growth and proliferation has been extensively emphasized [1,2]. Unlike normal cells, arginine is conditionally essential amino acid for the tumor cells proliferation. Arginine can be *de*

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novo synthesized from citrulline *via* active urea cycle [3] in normal cells, however, in tumor cells this pathway seems to be inactive due to lacks of expression of argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) [4,5]. ASS catalyze the condensation of L-citrulline and aspartic acid into argininosuccinate intermediate, while, ASL subsequently split this intermediate into arginine and fumaric acid [6]. The majority of tumor cells were authenticated to lack to the active ASS and ASL [7,8] with fails to regenerate arginine from citrulline *via* urea cycle, unlike to normal cells. Therefore, most of tumor cells are mainly dependent on dietary arginine to compensate their arginine requirements, so, deprivation of plasma arginine has a remarkable targeting effect on growth of tumor cells, with no effect to normal cells. Plasma arginine could be metabolized by the action of three key enzymes; arginase, arginine deiminase (ADI) and arginine decarboxylase [3,6]. The rationality of anticancer activity of ADI towards various types of tumors evolved from the lack of expression of ASS and ASL with inability to regenerate intrinsic arginine from citrulline [9–11].

Arginine deiminase (ADI, EC 3.5.3.6) is a guanidine-modifying enzyme catalyzing the irreversible deimination of arginine into citrulline and ammonia [12]. ADI has been identified with anticancer potency towards multiple of arginine-auxotrophic tumors [3]. The powerful anticancer activity of ADI has been extensively emphasized towards different melanoma and hepatocellular carcinoma lacking to the expression of ASS [9,13] ADI eliminates the intracellular plasma arginine, generating an arginine shortage in the ASS-deficient tumor cells, with no influence on normal cells that express ASS [5,14,7,1]. The lack of ASS expression in tumor cells was recognized mainly due to the aberrant CpG methylation of ASS promotors, in addition to other epigenetic transcriptional regulation [10].

ADI was characterized from different bacterial species belonging to the genera *Bacillus*, *Pseudomonas*, *Leuconostoc*, *Lactobacillus*, *Listeria*, *Clostridium* and *Mycoplasma* (reviewed in [17]. ADI of *M. arginini* was firstly recognized with its potential anticancer activity towards various tumor cell lines [5], however, the major challenges that restrict the *in vivo* application of this enzyme are its higher antigenicity, short half-life time and thermal lability [3,18,19,1]. Although, PEGylation of this enzyme greatly improves the pharmacokinetic properties, reduce the antigenicity, and stabilize the conformational structure of this enzyme [7], however, the thermal instability and susceptibility to proteolytic cleavage remain the major challenge for *in vivo* usage of this enzyme. ADI had been purified and characterized from thermotolerant *A. fumigatus,* displaying a plausible higher structural stability [17], comparing to bacterial ADIs. In addition, dextran has been frequently authenticated as non-toxic, non-immunogenic compound and potential stabilizer for the conformational structures of various therapeutic enzymes [20–23]. Thus, we have been motivated to further explore ADI from thermophilic fungi with prospective robust thermal, and catalytic stabilities, moreover, to assess the potentiality of ADI conjugation with dextran on strengthen its conformational stability and resistance to proteolytic cleavage.

2. Materials and methods

2.1. Materials

Dextran from *Leuconostoc mesenteroides* (200 kDa) and polyacrylamide solution were purchased from Sigma-Aldrich Co. (Spruce St. Louis, MO, USA). Protein ladder (Blue Plus Protein Maker, Cat # DM101, 14–100 kDa) was obtained from ThermoFisher Scientific, USA. All the other chemicals were of analytical grade. Four weeks old female mice were obtained from the animal breeding lab, Faculty of Veterinary, Zagazig University, Egypt. Animal experimental procedures were carried out according to the Guide of Institutional Animal Care and Use Committee (IACUC) at Faculty of Medicine, Zagazig University and confirmed to follow NIH guidelines. All the animal researches used in this study were approved by Zagazig University under protocol 15-08-263.

2.2. Purification, molecular subunit structure and peptide fingerprinting of *A. nidulans ADI*

Thermophilic *Aspergillus nidulans* was isolated, recognized with optimal growth at 45 °C, and potent ADI producer, identified based on morphological features and molecular identity of ITS sequence [24]. The isolate sequence has been deposited on Genbank with accession # MH633720.1, and at Assiut University Mycological Center (AUMC) with deposition # AUMC13375. *A. nidulans* was grown on Potato Dextrose Agar medium (BD Difco, Cat #213,400), incubated for 7 days at 45 °C. One plug of 7 days old cultures of *A. nidulans* was inoculated into 50 ml Potato Dextrose Broth medium (PDB, Cat #. DF0549-17-9) in 250 ml Erlenmeyer conical flak, incubated at 45 °C for 8 days. The mycelial pellets were collected, washed by sterile distilled water. Fifty grams of the fungal mycelia were pulverized in liquid nitrogen and dispensed in 100 ml of sterile 50 mM Tris−HCl of 1 mM PMSF, 1 mM EDTA and 1 mM 2-mercaptoethanol [25,26,22,17]. The homogenate was vortexed for 5 min, centrifuged at 6000 rpm for 10 min at 4 °C, the supernatant was used as the crude enzyme source for downstream purification processes. The enzyme was purified by gel-filtration and ion-exchange chromatography according to our published studies [23,27,22,28,29,24,30] based on the fractions activity and their molecular homogeneity by SDS-PAGE analysis.

The purified *A. nidulans* ADI was electrophoresed on SDS-PAGE, the putative ADI band was excised, eluted and subjected to trypsinization prior to peptide sequence analysis by LC−MS/MS [31,26,24]. The extracted MS/MS data were analyzed with Protein Pilot 4.0 (ABSCIEX) for peptide identification, normalizing to the proteome of *A. nidulans* [32].

2.3. Activity and concentration of Arginine deiminase

The activity of ADI was determined based on the amount of released citrulline according to [33] with slight modifications [23]. Briefly, the reaction mixture contains 10 mM L-arginine in Tris−HCl buffer (50 mM, pH 8.0), 1 mM $CaCl_2$ and 100 µl of enzyme preparation in 1 ml total volume. Blanks of enzyme and substrate were prepared. Th reaction was incubated at 37 °C for 30 min, stopped by 10% TCA, the mixture was centrifuged at 10,000 rpm for 5 min, the supernatant was used for citrulline assay by Carbidino reagent [34]. One unit of ADI was expressed by the amount of enzyme releasing 1 µmole of citrulline from L-arginine substrate per minute per mg protein under standard assay.

The protein content of enzyme was determined by Bradford's reagent using bovine serum albumin as standard [35].

2.4. Conjugation of *A. nidulans ADI* with Dextran, spectroscopic and molecular analyses

The purified ADI was crosslinked with sodium periodate-activated dextran [36] in presence of 15 mM trimethylaminoborane for 24 h at 4 °C. The developed Schiff base of ADI reactive amino groups and dextran aldehyde groups were stabilized by reduction with sodium borohydride [30]. Different molar ratios of purified ADI (0.039, 0.078 and 0.117 mM) and activated dextran (10, 20, 50 mM) were investigated. The conjugates of Dextran-ADI were purified from the unbounded dextran and free ADI by gel-filtration chromatography, the Dextran-ADI conjugates fractions were selected based on their molecular electrophoretic mobility on SDS-PAGE [21,37] comparing to native ADI. The activity of Dextran-ADI conjugate was assessed by the standard assay. Replica of SDS-PAGE gel was prepared, the 1st gel part was stained with 0.2% Coomassie brilliant blue R-250, while the 2nd part was stained with 0.4% periodate-fuchsin solution for glycoproteins conjugates assay [38]. The fractions of Dextran-ADI conjugates were collected and concentrated by 100 kDa dialyzer (Slide-A-Lyzer, Thermo-Scientific, USA). The yield of ADI immobilization (%) was expressed by the activity of ADI-conjugates to the free ADI x 100.

2.5. Modification of surface reactive groups of A. nidulans ADI upon Dextran conjugation

The surface reactive amino groups of ADI-dextran conjugates were assessed by Ninhydrin assay [39]. Briefly, the free and Dextran-ADI conjugates (same concentration 1.0 mg/ml) were boiled with 100 µl of Ninhydrin reagent for 5 min and the developed blue complex was measured at A_{575} nm. Buffer and activated dextran solution were used as baselines.

The modification of ADI surface reactive ε-lysine amino groups upon dextran conjugation were assayed by Fluorescamine assay [40]. The native and Dextran-ADI conjugates, at the same concentration, were mixed with 10% fluorescamine reagent [21], and mixture was excited at λ_{390} nm, and the emitted fluorescence was measured at λ_{475} nm) (Varian Cary Eclipse Fluorescence Spectrophotometer, Agilent). The arbitrary fluorescence units (AU) was expressed by the sample emitted fluorescence subtracted from the buffer and dextran solution.

The total surface reactive thiols of native and Dextran-ADI conjugates were determined by Ellman's reagent at λ_{412} nm [41].

The modification ratio was expressed by

2.6. Proteolytic mapping of the native and Dextran-ADI conjugates

The proteolytic patterns of both of ADI forms in response to proteinase K and trypsin were assessed *in vitro* [30]. The native and modified ADI (200 µg/ml) were incubated with proteinase K and Trypsin (10 µmol/mg/min) for 1 h at 37 °C, then the proteolytic activity was stopped by 1 mM PMSF, and the residual activities of ADIs were measured by the standard assay.

2.7. Spectroscopic and DLS analyses of native and Dextran-ADI conjugates

The FT-IR spectra of native and Dextran-ADI conjugates were assessed by Bruker FT-IR Spectrometer in range 400-4000 cm^{-1} with KBr pellets. The molecular sizes of native and Dextran-ADI conjugates were assessed by dynamic light scattering (DLS) (Nano-ZS, Malvern Ltd., Malvern, UK) using trypsin (23.5 kDa) in the same buffer as internal standard [26]. The molecular mass of target protein was determined from the inference of size (nm) and mass of standard.

2.8. Biochemical properties of the native and Dextran-ADI conjugates

The optimum temperature for activity of native and Dextran-ADI conjugates was evaluated by incubating the enzymatic reaction at 30, 37, and 45 °C, then measuring the enzymes activity by the standard assay. The thermal stability was assessed by preincubating the enzymes without substrate at 30, 37, and 45 °C, and then measuring their residual activities after 15, 30, 60 and 90 min of incubation by standard assay. The thermal kinetic parameters; half-life time ($T_{1/2}$) and thermal inactivation rate (Kr) were determined [42].

The effect of reaction pH (3.0–10.0) on activity of native and Dextran-ADI conjugates were investigated using 50 mM of citrate-phosphate buffer (pH 3.0–5.0), Tris − HCl (6.0–10.0). The pH stability was evaluated by preincubating the enzymes at different pHs for 2 h at 4 °C, then measuring their residual activities by standard assay as described above. The pH precipitation of native and modified ADI was determined by incubating the enzymes at pH range 3.0–10.0 for 24 h at 4 °C, centrifuging the mixture at 10,000 rpm for 15 min, then measuring the precipitated proteins by standard assay. The putative isoelectric point (*p*I), is the pH at which maximal protein precipitation was detected [43].

The effect of different inhibitors on activity of native and Dextran-ADI was assessed. The enzymes preparations were desalted by dialysis (Cat # 546-00051, Wako Chem., USA) against 50 mM Tris − HCl buffer (pH 8.0) of 1 mM EDTA. Different cations such as Ba^{2+}, Fe^{3+}, Ca^{2+}, Hg^{2+}, Fe^{3+}, Al^{3+}, Zn^{2+}, Na^{+}, Cu^{2+}, and Na^{+} were added to the enzymes at 1 mM final concentration, after 2 h of incubation at 4 °C, the substrate was added, and enzymatic activities were measured by the standard assay. The influence of suicide amino acid reactive analogues namely; hydroxylamine, guanidine thiocyanate, DTNB, MBTH, H_2O_2 and PMSF (1 mM final conc.) on the activity of native and Dextran-ADI conjugates were assessed. The mixtures were incubated for 2 h at 4 °C, then the enzymes residual activities were measured by the standard assay as described before.

2.9. Substrate specificity and kinetic parameters of the native and Dextran-ADI conjugates

The affinity of native and modified ADI to deaminate various L-forms of free amino acids such as asparagine, tyrosine, lysine, methionine, valine, cysteine, phenylalanine, ornithine, glycine and alanine, comparing to L-arginine as standard substrate at 10 mM final concentration. The affinity of native and modified ADI to citrullinate the peptidyl-arginine residues of various peptides and proteins such as Boc-Gln-Arg-Arg-MCA, yeast enolase, β-lactoglobulin, β-casein, α-lactalbumin, bovine serum albumin, fibrin and peroxidase was assessed [44]. The activities of native and Dextran-ADI conjugates for each substrate were assessed based on the amount of released ammonia and citrulline as described above. The most deiminated substrates were selected, and the enzymes kinetic parameters such as Michalis-Menten constant (K_m), maximum velocity ($Vmax$), turnover number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) were determined using GraphPad Prism Software Package (Graphpad Software Inc., La Jolla, CA, USA).

2.10. In vitro anticancer activity

The antiproliferative activity of native and Dextran-ADI conjugates was evaluated towards different tumor cell lines; liver carcinoma (HepG-2), breast carcinoma (MCF-7), and colon carcinoma (HCT8, HT29, DLD1, and LS174 T) with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [45]. The 96-well plate was seeded with 10^3 cells/ well, incubated overnight at 37 °C in CO_2 incubator, then amended with different concentrations of each enzyme, and re-incubated for 48 h at the same conditions. MTT reagent (25 µl) was added, incubated for 2 h, and the intracellular formazan complex was dissolved in 100 µl DMSO, and the developed purple colored formazan complex was measured at λ_{570} nm. The percentage of cell viability was expressed by the A_{570} nm of sample normalizing to negative control x100. IC_{50} value was expressed by ADI activity that reduces 50% of initial number of tumor cells normalizing to phosphate buffered saline as baseline.

2.11. Pharmacokinetics of native and Dextran-ADI conjugates

The pharmacokinetic properties of native and Dextran-ADI conjugates were determined *in vivo* using male mice (25 g of 30 days old). The animal experiments were conducted according to the guidelines of Institutional Animal Care and Use Committee (IACUC) at Faculty of Medicine, Zagazig University, and confirmed to NIH guidelines under protocol 15-08-263. The mice were acclimatized for 5 days at controlled conditions. Single dose (100 µl) of the ADI and Dextran-ADI (8.0 µmol/mg/min) was intravenously injected to the tail vein of mice and acclimated for 20 days. Each treatment group has five mice. Normal mice and primate plasma of injected mice were used as negative and positive controls, respectively. Blood samples were collected intervally to assess the pharmacokinetic, biochemical and hematological parameters. The activity of native and Dextran-ADI conjugates was assessed in serum by the standard assay, based on the amount of released citrulline [33]. To evaluate the cytotoxicity of ADIs, elected biochemical parameters such as ALT, AST, ALP, total protein, albumin, globulin, urea and creatinine were assessed (Spectrum Co., Egypt).

Plasma L-arginine and L-citrulline titers were determined by HPLC

Table 1
Overall purification profile of Arginine deiminase from *A. nidulans*.

	Total protein (mg)	Total activity	Specific activity (μmol/mg/min)	Purification fold	Yield
Crude ADI	16.10	42.4	2.46	1	100
Acetone precipitate	1.85	17.5	9.49	3.85	41.4
Sephadex G_{200}	1.33	16.58	12.4	5.04	39.2
DEAE-Sepharose	0.59	7.6	12.9	5.3	17.91

(HPLC system E-Chrom Tech Model LC 1620A, P1620A C18; Shodex C18-120-54), with flow rate 1 ml/min. The mobile phase was 0.1% orthophosphoric acid in ultrapure deionized water (25:75 v/v). Arginine and citrulline were detected at λ_{195} nm, comparing to their standard concentrations. The peaks of arginine and citrulline were clearly compensated. Negative and positive controls were used as baselines.

2.12. Statistical analysis

All experiments were conducted in biological triplicates and the results were expressed by mean ± STDEV. The data were analyzed by one-way ANOVA with Fisher's Least Significant Difference of post hoc test (https://www.easycalculation.com/statistics/fishers-lsd-calculator.php).

3. Results

3.1. Purification, molecular subunit structure and peptide fingerprinting of *A. nidulans ADI*

ADI was purified from the mycelia of *A. nidulans* by acetone precipitation, gel-filtration and ion-exchange chromatography [24]. By the last purification step, the overall activity of ADI was increased by ˜5.3 folds with 17.9% yield (Table 1). The specific activity and concentration of purified *A. nidulans* ADI were 12.9 μmol/mg/min and 0.59 mg/ml, respectively. The molecular subunit structure of purified *A. nidulans* ADI was 48 kDa as revealed from the denaturing-PAGE (Fig. 1A), while the entire molecular mass as revealed from gel filtration by Sephadex G_{200} and native-PAGE analyses was about 120 kDa (Data not shown), suggesting the homotrimeric identity of purified *A. nidulans* ADI. The putative ADI from SDS-PAGE was excised and in-gel sequenced by LC–MS/MS (Fig. 1B). The recovered peptide sequences were annotated by alignment with ADI sequences from different sources (Fig. 1B). From the multiple alignment analysis, the partial peptide sequence of *A. nidulans* AD had a 60% similarity with ADI of *Mycobacterium avium* (WP009954974.1), *M. tuberculosis* (NP215517.1), and *Rhodococcus* sp (WP009478910), with 85% sequence coverage. The conserved active sites domains of the partially peptide sequence of *A. nidulans* ADI (Glu^{95}-Gly^{96}-Gly^{97}) was identical to ADI from species of *Mycobacterium*, *Streptomyces*, *Pseudomonas*, *Streptococcus*, *Clostridium* and *Lactobacillus* (Fig.1C). The phylogenetic analysis of partial peptide sequence of *A. nidulans* ADI with the database deposited sequences of ADIs was shown (Fig. 1D). From the phylogenetic analysis, four categories of ADIs were committed based on their sequence homology, *Streptococcus* spp (Clade I), *Bacillus* spp (Clade II), *Pseudomonas* spp (Clade III) and *Mycobacterium* spp (Clade IV). The peptide sequence of *A. nidulans* ADI has a higher proximity to the *Mycobacterium* spp clade (IV).

3.2. Dextran-ADI conjugation, molecular and kinetic parameters

The purified *A. nidulans* ADI was conjugated to dextran- reactive aldehyde groups *via* the formation of aldimine linkage followed by subsequent reduction. The scheme of dextran activation and conjugation of ADI was shown (Fig. 2A). Dextran-ADI conjugates were purified from the unbounded active dextran, and native ADI by gel-filtration chromatography using Sephadex-G_{200} column, the fractions of Dextran-ADI conjugates were selected based on their activities and molecular electrophoretic mobility on SDS-PAGE (Fig. 2B). The gel of native ADI was stained regularly with Coomassie brilliant blue, while Dextran-ADI conjugate was stained with periodic acid-Schiff's reagent (Fig. 2B). Different molar ratios of ADI and activated dextran have been evaluated to obtain the maximum conjugation yield and specific activity. Obviously, the activity of ADI was increased exponentially with increasing the enzyme molar ratio regarding to dextran concentration. The maximum specific activity (9.8 μmol/mg/min) was obtained using 0.078 mM of ADI and 20 mM activated dextran (Table 2), with 77.8% conjugation yield and overall increase to molecular mass to ˜100 kDa, as revealed from denaturing-PAGE analysis. Theoretically, each surface lysine moiety of ADI could be covalently attached to glucose residue of

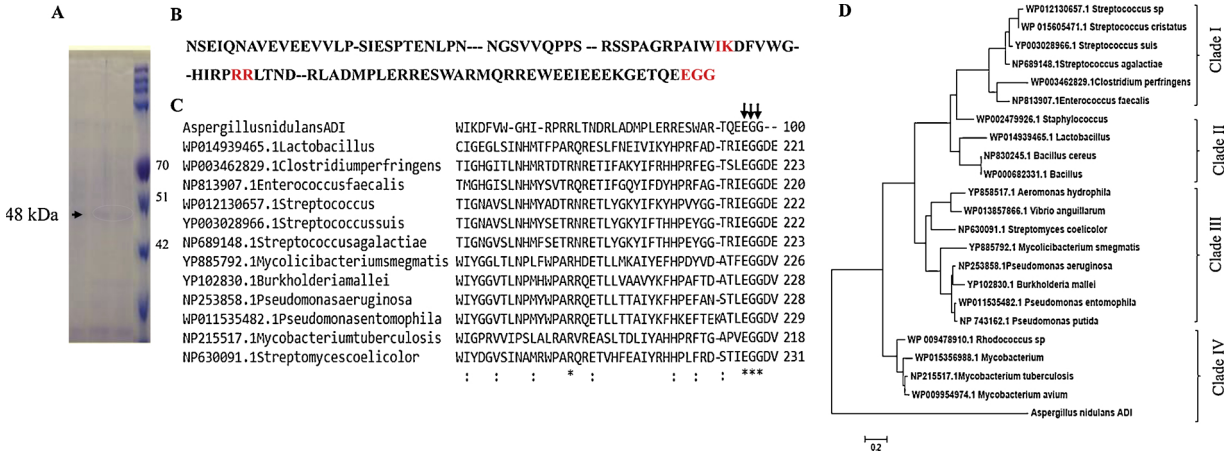

Fig. 1. Molecular subunit structure of the purified *A. nidulans* ADI. The pellets of *A. nidulans* were collected, ADI was extracted, purified by gel-filtration and ion-exchange chromatography, and its amino acid sequence were analyzed by LC–MS/MS. (A) SDS-PAGE analysis of purified *A. nidulans* ADI, M; protein ladder. (B) LC–MS/MS analysis of *A. nidulans* ADI, peptide sequence annotated by alignment with ADI sequences from different sources. Conserved active site domains of *A. nidulans* ADI aligned with ADI sequences. (D) Phylogenetic analysis of *A. nidulans* ADI sequence with the database deposited proteins.

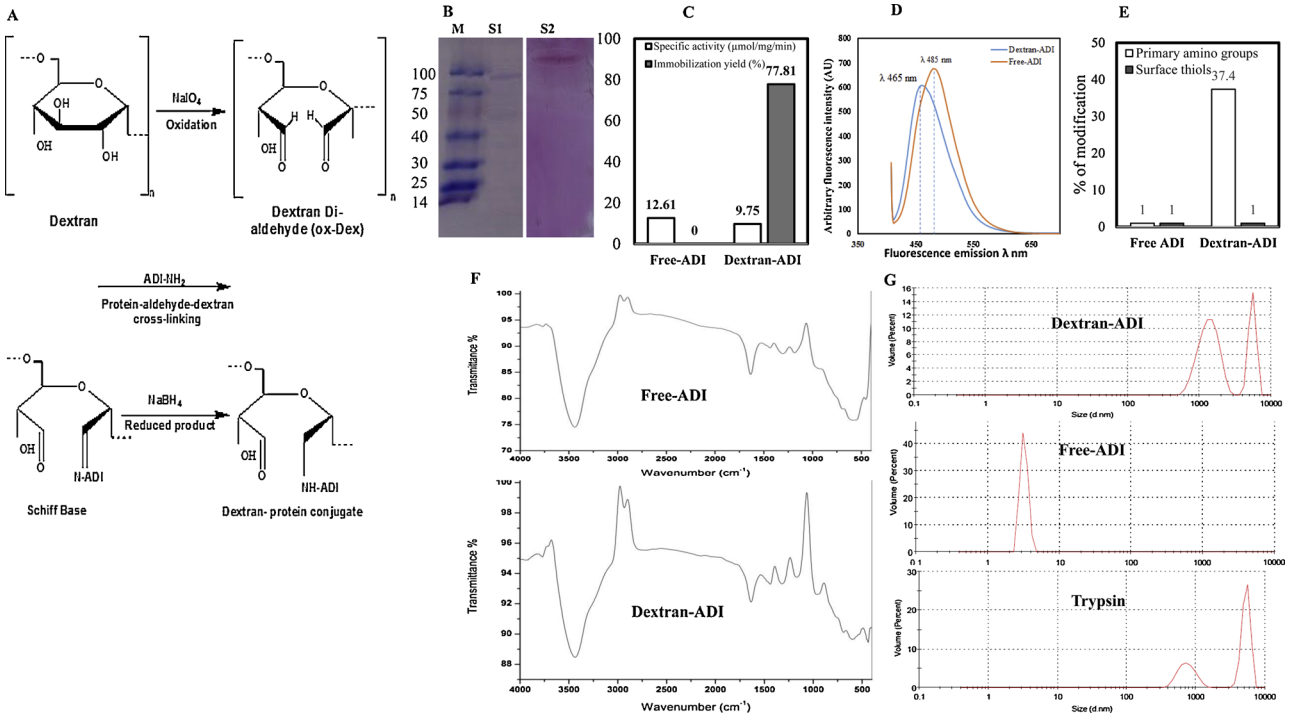

Fig. 2. Dextran activation, covalent conjugation, kinetic properties and structural stability of Dextran- ADI conjugates comparing to free enzyme. Scheme of dextran activation and covalent conjugation with ADI, dextran was activated by sodium periodate to form reactive aldehyde groups enabling to form Schiff base with the ADI surface reactive amino groups, with subsequent reduction by sodium borohydride (A). SDS-PAGE electrophoretic mobility of free and dextran-ADI conjugates from the Sephadex-G200 column (B, left panel stained with Coomassie brilliant blue (S1), right panel stained with Fuchsin solution (S2)), M, is protein ladder (Protein ladder (Blue Plus Protein Maker, Cat # DM101, 14–100 kDa). Activity and immobilization yield (C), modification of surface reactive ε-amino groups of lysine by fluorescence assay (D), reactive amino groups of amide amino acids and thiols (E), FT-IR analysis (F), and molecular size by DLS analysis (G) for the native and Dextran-ADI conjugates.

dextran molecule by Schiff base, which subsequently was reduced to amide bond under alkaline conditions. Thus, the molar ratio optimization of dextran to ADI is a technical critical issue, since, over conjugation of ADI with dextran might have a negative effect on the enzyme tertiary/ catalytic structure, by tackling the formation of enzyme-substrate complex. Thus, at molar ratio 20:0.078 of dextran to ADI, dextran residues may have the lowest steric hinderance towards substrate active site complex formation. The highest activity of Dextran-ADI conjugates was 9.7 comparing to 12.61 μmol/ mg/ min of native ADI with conjugation yield 77.81%. The modification of surface reactive lysine groups of ADI upon dextran conjugation has been estimated by fluorescamine assay. Since, the ε-amino group of lysine of ADI is highly reactive, forming amid bond with the aldehyde group of dextran. From the scanning emission spectra, the maximum emitted fluorescence was reported at λ_{475} nm, the emitted fluorescence of dextran-ADI was reduced by 30% comparing to free enzyme, ensuring the implication of ˜30% of surface lysine residues of ADI with dextran (Fig. 2 D). Meanwhile, the modification of ADI surface reactive amines of amide amino acids (glutamine and asparagine) was about 37.4% upon dextran conjugation (Fig. 2E), as revealed from Ninhydrin assay. However, there was no modification on surface ADI thiols, approving the lack of interaction of dextran with the enzyme surface cysteine and methionine residues. Thus, from the inspection of surface reactive amino groups and thiols, it could be hypothesized that conjugation is mainly due to interaction with ε-amino groups of lysine residues, relatively with surface amid amino acids, and no interaction with surface thiols of ADI.

To assess the development and shifting of the functional groups of ADI upon conjugation with dextran, FT-IR analysis was conducted to native and Dextran-ADI under the same conditions. From the FT-IR chromatogram (Fig. 2F), a slight shifting on the N—H groups from 2359 to 2362 cm and N—C groups from 1315 to 1320 cm for ADI upon conjugation with dextran, suggesting the deprotonation of NH group and amid bond formation. As well as, the molecular mass of ADI upon dextran conjugation was assessed by Zeta-Sizer DLS analysis normalizing to trypsin (25.5 kDa) as authentic protein. From the chromatogram of DLS, an obvious increase to ADI molecular mass upon dextran conjugation as revealed from the distinct shifting on molecular size (Fig. 2G).

Table 2
Conjugation kinetic parameters of different molar ratio of purified *A. nidulans* ADI with the activated dextran.

ADI Modifications			Kinetic Parameters		
Dextran (mM)	ADI (mM)	Specific activity (μmol/ mg/min)	Conjugation yield (%)	Average surface modification (%)	Molecular weight subunit (kDa)
–	0.039	7.5	–	–	˜ 50
	0.078	12.6	–	–	˜ 50
	0.117	11.4	–	–	˜ 50
10	0.039	4.0	53.4	10	˜ 55
	0.078	7.6	60.4	14	˜ 55
	0.117	7.9	69.3	19	˜ 55
20	0.039	4.5	60	30	˜ 85
	0.078	9.8	77.8	38	˜ 100
	0.117	8.3	72.9	39	˜ 100
50	0.039	4.9	65.4	38	˜ 120
	0.078	8.8	69.9	42	˜ 120
	0.117	8.0	70.2	48	˜ 120

3.3. Resistance of Dextran-ADI conjugates to in vitro proteolysis

The blocking of surface proteolytic recognition sites on the surface

of ADI upon conjugation with dextran was assessed. The native ADI and Dextran-ADI conjugates at the same concentration (0.1 mg/ml) were incubated with proteinase K and trypsin (10 μmol/mg/min) in total volume 1 ml at 37 °C for 2 h. The residual activities for both enzymes were determined, intervally, by the standard assay. The Dextran-ADI conjugates displayed about 2.5 folds more resistance to proteolysis by proteinase K and trypsin for 60 min, compared to native-ADI, suggesting the shielding of about 50% of ADI surface recognition sites for proteases. The native and Dextran-ADI conjugates retained about 25% and 72% of their initial activities in response of proteinase K treatment for 60 min. However, upon dextran conjugation, ADI retains about 90% of its initial activity after 60 min of proteolysis with trypsin (Fig. 3A, B). So, ADI is more sensitive to proteolysis with proteinase K than trypsin by about 1.5 folds, suggesting the presence of multiple recognition sites for proteinase K on ADI surface than for trypsin. Theoretically, these results were confirmed from *in silico* prediction of proteolytic sites by peptide cutter (ExPASY, bioinformatics Resource Portal). The proteolytic map of ADI in response to proteinase K and trypsin were shown (Fig. 3C, D) as extracted from the peptide cutter portal. Practically, the putative number of proteolytic cleavage sites on surface of ADI were found to be 48 and 11 sites for proteinase K and trypsin, respectively (Fig. 3E). The higher proteolysis of ADI upon incubation with proteinase K for 60 min being reasonable, since this enzyme attacks multiple recognition sites on ADI surface. Practically, the native ADI had a more resistance to cleavage by trypsin than proteinase K that might be due to lower frequency of recognition sites for trypsin. The eleven putative recognition sites for trypsin on ADI were reported among the consecutive amino acids; arginine-serine, lysine-aspartic acid, arginine-arginine, arginine-leucine, arginine-aspartic acid, arginine-methionine, arginine-glutamic acid and lysine-glycine. Among these sites, two ε-amino groups of lysine residues (about 20%), are highly reactive to form Schiff base with the aldehyde group of the active glucose of dextran.

3.4. Biochemical properties of the native and Dextran-ADI conjugates

The biochemical properties of native and Dextran-ADI conjugates such as reaction temperature, thermal stability, reaction pH, pH stability, in addition to influence of inhibitor and activators were estimated. From the reaction temperature profile (Fig. 4A), the native and conjugated enzyme have the same catalytic profile to reaction temperature. The maximum activities of native and Dextran-ADI were recorded at 37 °C after 60 min of incubation, with obvious stability for enzymes activities with further incubation time. After 60 min incubation at 30 °C and 45 °C, the activities of both enzymes were reduced by about 30% comparing to their activities at 37 °C. The thermal stability of native and Dextran-ADI conjugates was assessed at 4, 30, 37 and 45 °C (Fig. 4 B,C). Obviously, Dextran-ADI conjugates had more thermal stability at all the tested temperature degrees. The thermal kinetic parameters for native and Dextran-ADI conjugates were summarized in Table 3. The half-life time ($T_{1/2}$) of Dextran-ADI was plausibly increased by about two folds at each experimented temperature. At 4 °C, the $T_{1/2}$ of Dextran-ADI (2440 h) was increased by about 1.6 folds comparing to native ADI (1445 h), with significant decreasing to the thermal denaturation rate of Dextran-ADI ($0.\ 2 \times 10^{-6}$) by approximately 10 times comparing to the native ADI. At 37 °C, the $T_{1/2}$ of ADI was increased by about two folds upon conjugation with dextran (34.2 h) comparing to the native ADI (17.3 h). The structural catalytic stabilities of ADI upon conjugation with dextran were increased by about 1.72, 1.98 and 2.48 folds at 30, 37 and 45 °C, respectively.

The activity of native and Dextran-ADI conjugates in response to reaction mixture pH (3–10) was assessed, under the standard conditions. From the results (Fig. 4D), the native and Dextran-ADI conjugates had the same catalytic response to reaction pH. The highest activity for both enzymes was measured at pH 7.0–8.0, with significant reduction to their activities at pH 3.0 and pH 10.0. Practically, dextran conjugation had no effect on the ionic structure of ADI and kinetics of binding with substrates. The effect of pH (4.0–9.0) on enzyme precipitation was assessed by incubating the native and Dextran-ADI at the same concentrations for 24 h at 4 °C, centrifugation and measuring the amount of precipitated protein by standard assay. The highest precipitated proteins were measured at pH 4.5–5.5 (Fig. 4E), hypothesizing the putative isoelectric points of these enzymes at this pH, negating the change on ADI charge upon dextran conjugation. The pH stability of native and Dextran-ADI conjugates was evaluated by preincubating at different pHs for 2 h at 4 °C, then measuring their residual activities. Both of ADIs have the same pH stability profile in response to different pHs (Fig. 4 F), with maximum activity at pH range 6.5 - 8.5. Taken together, the reaction pH, precipitation pH and pH stability clearly negate the potential negative impact of covalent conjugation of dextran residues on ADI tertiary structure and ionic state.

3.5. Effect of inhibitors and amino acid suicide analogues on native and Dextran-ADI conjugates

The catalytic and structural identities of free and Dextran-ADI conjugates were shaped in response to various inhibitors and amino acids suicide analogues. Prior to the addition of inhibitors, the enzymes

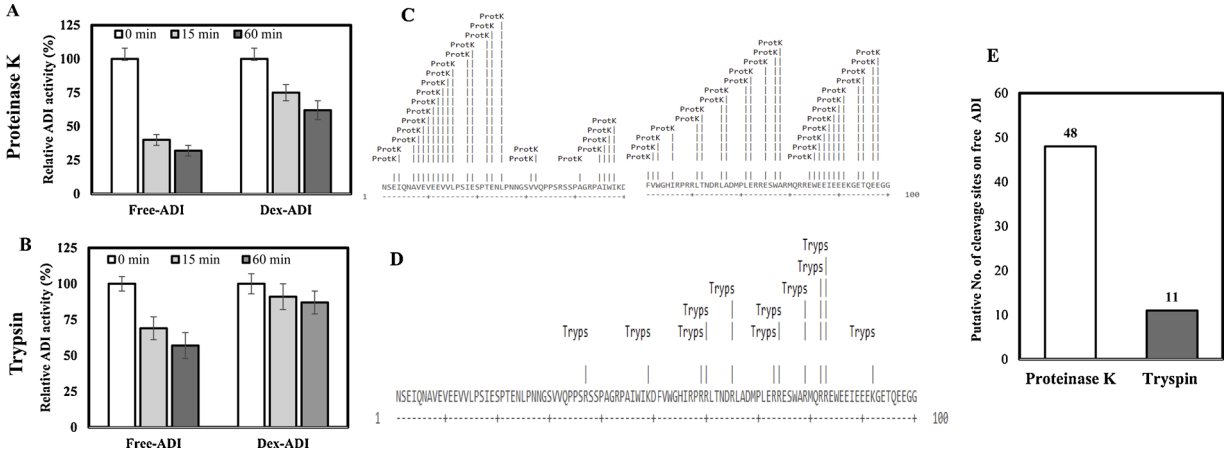

Fig. 3. Catalytic activity and structural stability of free *A. nidulans* and Dextran-ADI conjugates in response to proteolytic cleavage by proteinase K and trypsin. The free and Dextran-ADI conjugates were digested by proteinase K and trypsin for 15 and 60 min, and their relative activities were evaluated (A, and B). The putative proteolytic maps of *A. nidulans* ADI peptide fragments from the ExPASy portal (https://web.expasy.org/peptide_cutter) in response to cleavage by proteinase K and trypsin are shown (C, D) and the putative number of cleavage sites (E).

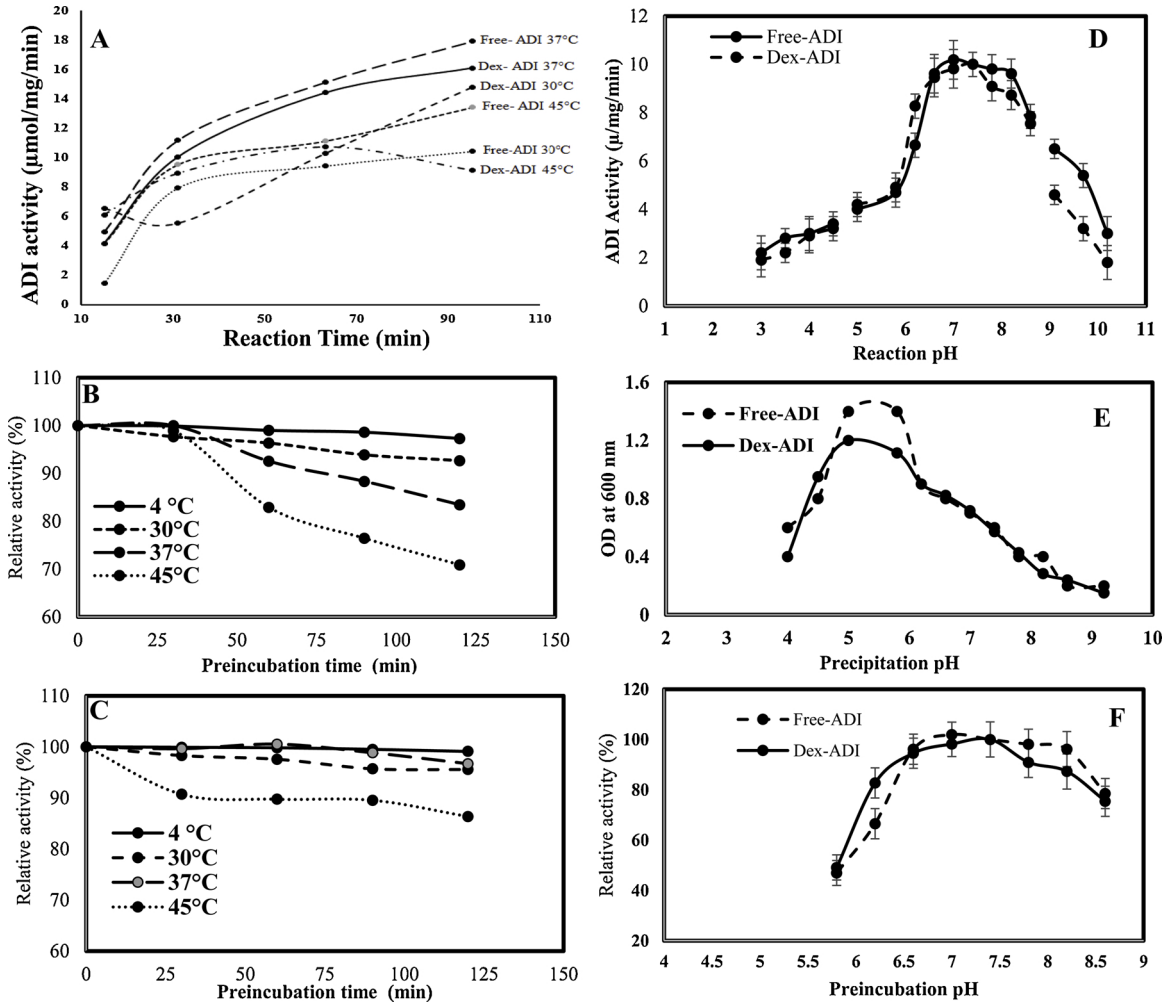

Fig. 4. Biochemical properties of *A. nidulans* free and Dextran-ADI conjugates. A, The influence of reaction temperature on the activity of free and Dextran-ADI conjugates. Thermal stability of free-ADI (B), Dextran-ADI conjugates (C). Reaction pH (D), precipitation pH (E) and pH stability of free and Dextran-ADI conjugates (F).

Table 3
Thermal kinetic parameters of ADI from and *A. nidulans.*

H	Free-ADI		Dextran-ADI		Stabilizing Ratio (%)
	$T_{1/2}$ (h)*	K_r (min)*	$T_{1/2}$ (h)	K_r (min)	
4	1445	0.02×10^{-4}	2440	0.002×10^{-4}	1.69
30	23.5	0.12×10^{-4}	40.2	0.01×10^{-4}	1.72
37	17.3	0.176×10^{-4}	34.2	0.04×10^{-4}	1.98
45	3.4	0.2×10^{-4}	8.4	0.08×10^{-4}	2.48

ln (At/A0) = - Kr *Tm* where A_0 and A_t are the specific activity of ADI at zero and *t* time.

*Half-life time ($T_{1/2}$) was expressed by time which the enzyme retains 50% of its initial activity by preheating without substrate at each temperature degree.

**Thermal denaturation rate (Kr) was expressed by the logarithmic decreasing of enzyme activity with the time at each temperature. It described by the first-ordered kinetic model.

were demetallized by dialysis against 50 mM Tris − HCl of 1.0 mM EDTA. Dialyzing the native and Dextran-ADI indicated the retention of about 40–50% of their initial activities, ensuring their metalloproteineous identity. The catalytic identities of enzymes were completely restored upon addition of KCl at 1 mM (Table 4), while a slightly positive effect by $BaCl_2$ `on activity of both enzymes. No obvious increasing on activity of apo-ADIs was noticed with addition of divalent cations such as Ba^{+2}, Mg^{+2}, Hg^{+2}, Ca^{+2}, Zn^{+2} and trivalent cations Fe^{+3} and $Al^{+3,}$ normalizing to the residual activity of demetallized ADIs. Thus, the strong positive effect of monovalent cation K^+ on restoring the activity of both ADI forms, authenticate their dependency on K^+ ions as cofactor.

The active sites and catalytic domains of native and Dextran-ADI conjugates were plotted from the amino acid suicide analogues such as hydroxylamine, Iodoacetate, 6-Diazo-5-Oxo-Norleucine, Guanidine thiocyanate, DTNB, MBTH, H_2O_2 and PMSF. Overall, chemical conjugation of ADI with dextran had a slightly stabilizing effect on the enzyme catalytic structure. The residual activity of ADI upon conjugation with dextran has been obviously increased compared to native ADI for all inhibitors (Table 4). The activity of Dextran-ADI conjugates was higher than the native one by about 2 folds in response to oxidizing effect by H_2O_2. Similarly, the activity of native ADI was reduced by 2 folds comparing to dextran conjugated ADI, in response to MBTH as specific reactive inhibitor to the surface primary amino groups. The dextran-ADI conjugates and native one retains 24.5% and 2.3% of their initial activities, respectively, upon addition of hydroxylamine, revealing the stabilizing effect of dextran moieties on surface lysine residues. The activity of native ADI was reduced by about 9.5 folds than Dextran-ADI conjugates by DTNB addition, assuming the shielding of L-cysteine residues from oxidation by DTNB. Overall, dextran conjugation partially shields the surface reactive lysine and cysteine residues of ADI from oxidation by the tested compounds.

Table 4
Relative activity of the native and Dextran-ADI in response to inhibitors and amino acid suicide analogues.

	Free-ADI	Dex-ADI
Control	100	100
Apo-ADI	40	50
KCl	98	97
$BaCl_2$	58.8	61.2
$MgCl_2$	40.3	49
$HgCl_2$	13.4	40
$FeCl_3$	50	45
$AlCl_3$	48	47
$CaCl_2$	51.9	45.9
$ZnCl_2$	10	12
NaCl	11	13
K_2CrO_4	36	42
Urea	36	46.5
CuSO4	43.4	33
$NaIO_3$	34	41
Na_2WO_4	26.9	30
Hydroxylamine	2.3	24.5
Iodoacetate	2.6	23.9
6-Diazo-5-Oxo-Norleucine	1.5	10.1
Guanidine thiocyanate	0.9	30.6
DTNB	4.9	46.7
MBTH	2.6	20.5
H_2O_2	2.9	25.8
PMSF	1.2	1.1

The final concentration of these compounds was adjusted to 1 mM on the reaction mixture.

3.6. Kinetics and catalytic properties of native and Dextran-ADI conjugates

The affinity of native and Dextran-ADI conjugates to deaminate/deiminate free amino acids and peptidyl arginine residues regarding to free L-arginine as substrate, was assessed based on released ammonia and citrulline. From the results, dextran conjugation had a little effect on the catalytic properties of ADI towards free amino acids and peptidylarginine residues. Based on citrullination assay, the relative activities of native and Dextran-ADI were ranged from 10 to 30% towards all the free amino acids, normalizing to L-arginine as substrate. Interestingly, upon dextran conjugation, dramatic decreasing to ADI activity to all the tested peptidylarginine residues was determined, comparing to the native ADI (Table 5), this shift might be due to the stereo-conformational change on ADI by dextran residues, subsequently influencing on its catalytic efficiency. The fluctuation on activity of ADI and Dextran-ADI conjugates towards the peptidylarginine residues could be correlated to the number and orientation of arginine residues "on surface or deeply embedded on ADI core". The relative deiminating activity of native and dextran-ADI to peptidylarginine residues of Gln-Arg-Arg-MCA was 60% and 12%, respectively, normalizing to free L-arginine as standard substrate. So, upon dextran conjugation, the activity of peptidylarginine deiminase of ADI was reduced by about 5 folds comparing to the native enzyme. As well as, the activity of Dextran-ADI to deiminate bovine serum albumin was reduced by 12 folds comparing to native enzyme. Also, the activity of ADI was completely abolished towards fibrin and dehydrogenase upon conjugation with dextran, ensuring the structural and catalytic improvements of ADI properties upon dextran conjugation. The deaminating affinity of native and Dextran-ADI to free L-arginine was 40% and 15%, respectively.

The kinetic properties of native and Dextran-ADI for peptidylarginine residues of Boc-Gln-Arg-Arg-MCA and free L-arginine were determined based on released citrulline and ornithine (Table 6). Kinetically, both ADIs displayed a significant affinity to deiminate free L-arginine, followed by Boc-Gln-Arg-Arg-MCA, with minor deaminating activity to L-arginine. The affinity (K_m) of native and Dextran-ADI conjugates to deiminate free L-arginine was 4.8 and 0.92 mM, while to deiminate Boc-Gln-Arg-Arg-MCA was 12.3 and 9.5 mM, respectively.

Table 5
Substrate specificity of the purified *A. nidulans* free and Dextran-ADI conjugate.

	Substrate	Arginine residues No.	Substrate Conc. (mM)	Native-ADI Relative activity (%)	Dextran-ADI Relative activity (%)
Free Amino Acids	L-Arginine	1	10	100	100
	L-Asparagine	–	10	5	8
	L-Tyrosine	–	10	11	19
	L-Lysine	–	10	10	0
	L-Methionine	–	10	5	10.4
	L-Valine	–	10	5	8
	L-Cystine	–	10	5.8	10
	L-Phenylalanine	–	10	32.3	29
	L-Ornithine	–	10	15	9
	L-Glycine	–	10	10	2.9
	L-Alanine	–	10	10	17.9
	L-Tryptophan	–	10	18	21
Proteins	Boc-Gln-Arg-Arg-MCA	2	10	60	12
	Yeas enolase	14	12	26	9
	β-Lactoglobulin	3	12	20	4
	β-Casein	5	10	28	5
	α-Lactalbumin	1	20	30	10
	Bovine serum albumin	26	4	48	4
	Dehydrogenase	29	3	24	3
	Fibrin	7	10	45	0
	Peroxidase	14	8	42	25

Thus, upon dextran conjugation, the ADI affinity to deiminate free L-arginine was increased by ~ 5.3 folds, however, its affinity to deiminate Boc-Gln-Arg-Arg-MCA was reduced by about 25%. Additionally, the deaminating affinity of ADI to free L-arginine was dramatically reduced by about 3 folds upon dextran conjugation. The catalytic efficiency (K_{cat}/K_m) of Dextran-ADI conjugates (2.6 $mM^{-1}s^{-1}$) was increased by ~ 2.4 folds comparing to native ADI (1.1 $mM^{-1}s^{-1}$) to deiminate free L-arginine. The catalytic efficiency of Dextran-ADI to deiminate free L-arginine (2.6 $mM^{-1}s^{-1}$) was increased by ~ 86 folds than its deaminating activity (0.03 $mM^{-1}s^{-1}$).

3.7. In vitro anticancer activity of native and Dextran-ADI conjugates

The antiproliferative activity of native and Dextran-ADI towards the different cell lines; breast (MCF-7), liver (HepG-2) and colon cancer (HCT8, HT29, DLD1 and LS174 T) cell lines was assessed, using phosphate buffered saline as negative controls. From the results (Fig. 5A-E), the antiproliferative activity of ADI had been increased by about 15–18 % towards all the tested liver, breast and colon cancer cell lines, upon conjugation with dextran, authenticating the positive effect of dextran moieties on improving and stabilizing the catalytic and conformational structures of ADI. The viability of tumor cells was evaluated based on the IC_{50} values of each enzyme, after 48 h of incubation (Fig. 5G). The IC_{50} values for native-ADI were 6.3, 5.6, 6.3, 9.4, 12.5 and 12.5 μmol/mg/min for HepG-2, MCF-7, DLD1, HT29, LS174 T and HCT8, while for Dextran-ADI were 4.7, 4.6, 3.8, 6.4, 9.7 and 10.5 μmol/mg/min, respectively. Obviously, ADIs exhibited a higher antiproliferative activity against liver and breast cancer lines than the colon cancer cell lines (HT29, LS174 T and HCT) except the colon cancer cell DLD1. The significant antiproliferative activity by Dextran-ADI was exhibited against colon cancer DLD1 (IC_{50} 3.82 μmol/mg/min), revealing the metabolic differences among the tested colon cancer cell lines. The sensitivity of liver and breast cancers to external arginine deprivation, ensures the highly inactivation of their intrinsic ASS, comparing to colon cancer cell lines. The antiproliferative activity of native and chemically modified ADI from different microbial sources, against different cancer cell lines have been summarized in Table 7.

Table 6
Kinetic parameters of the purified ADI from *A. nidulans*.

Substrate	Native-ADI				Dextran-ADI			
	K_m(mM)	V_{max}(μmol/mg/min)	K_{cat} (s^{-1}) x 10^{-3}	K_{cat}/K_m($_{mM}s^{-1-1}$) x 10^{-3}	K_m(mM)	V_{max}(μmol/mg/min)	K_{cat} (s^{-1})x 10^{-3}	K_{cat}/K_m($_{mM}s^{-1-1}$) x 10^{-3}
Boc-Gln-Arg-Arg-MCA	12.3	24.3	2.0	0.4	9.5	33.2	2.6	0.8
Free L-Arginine*	4.8	59.5	6.2	1.1	0.92	96.7	10.2	2.6
Free L-Arginine**	36	15.2	0.6	0.03	20.8	12.4	0.33	0.03

*The activity assay was expressed by the amount of released citrulline.
**The activity assay was expressed by the amount of released ornithine.

3.8. *In vivo* pharmacokinetic and cytotoxic properties of native and Dextran-ADI conjugates

The pharmacokinetic properties of native and Dextran-ADI conjugates and biochemical properties of mice were determined intervally using single dose of enzymes (10 μmol/mg/min. The biological half-life time ($T_{1/2}$) of native and Dextran-ADI was 50.14 h and 66.8 h, respectively, thus, upon dextran conjugation the catalytic activity of ADI was increased by 1.7 folds (Fig. 6). The obvious increasing on the biological half-time of ADI *in vivo* upon dextran conjugation, ensuring the acquired catalytic stability of ADI.

The toxicity of ADIs in mice was assessed based on the biochemical and hematological parameters of single enzyme dosing. Blood samples were collected intervally, the titer of different biochemical parameters was determined. From the biochemical profile (Table 8), the titer of liver enzymes ALT, AST and ALP were obviously increased by about 2–3 folds in response to the both forms of ADI, particularly after 24 h of injection, however, after 48 h, the activities of these enzymes were suppressed to be close to the normal titers. Nevertheless, the native and Dextran-ADI did not display any significant effect on the titers of total proteins, albumin, globulin, A/G ratio, urea and creatinine along the experimented time points (4, 24 and 48 h), normalizing to negative controls. In addition, the hematological parameters of mice in response to native and Dextran-ADI treatment were determined. As well as, the ADIs had no effect on the tested hematological parameters such as WBCs, RBCs, HGB and platelets, comparing to negative controls (Data not shown). Thus, as revealed from biochemical and hematological parameters, ADI and dextran ADI conjugates had no signs of toxicity on the experimental animals.

The concentration of L-arginine and citrulline in sera of mice responsive to injection of native and Dextran-ADI conjugates were determined by HPLC (Fig. 6). After 24 h, the concentration of L-arginine was reduced by 30% and 84% (Fig. 6C) in response to injection of the native (4.25 μg/ml) and Dextran-ADI (1.1 μg/ml), respectively, comparing to the negative control mice (6.14 μg/ml). Remarkably, the titer of citrulline in animal sera was increased by 1.5 and 3.8 folds in response to native (3.16 μg/ml) and Dextran-ADI (8.2 μg/ml) treatment, respectively, comparing to the control (2.15 μg/ml). Thus, upon dextran conjugation, the catalytic activity of ADI to deiminate L-arginine into citrullinate, was increased by about 2.6 folds comparing to native ADI *in vivo*.

4. Discussion

The antiproliferative activity of ADI has been extensively emphasized against different cancer cells that lacks to complete ASS

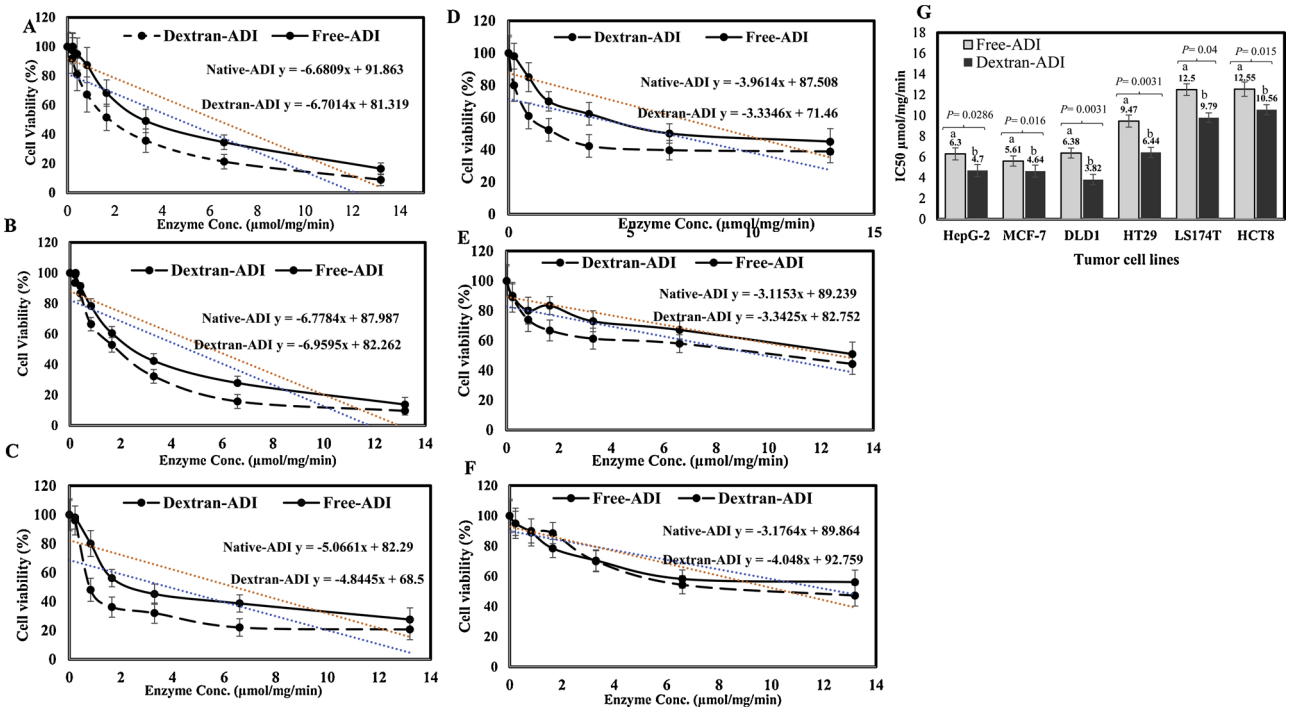

Fig. 5. *In vitro* anticancer activity of the native and dextran-ADI conjugates towards various cell lines; HepG-2 (A), MCF-7 (B), DLD1 (C), HT29 (D), LS174 T (E) and HCT8 (F). The IC50 value was expressed by the enzyme concentration suppressing the proliferation of the tumor cells by 50% normalizing to the negative controls (G). The results of IC_{50} values of enzyme towards each cell lines were subjected to analysis of variance (One Way ANOVA, Tukey HSD test at $p \leq 0.05$) using Costat software package. LSD value was calculated as described by Fisher (1950) using the same program.

Table 7
IC_{50} values of native and chemically modified ADI conjugates towards various cancer cell lines.

Cell line	Source	Free-ADI (μmol/mg/min)	Dex-ADI (μmol/mg/min)	PEG-ADI (μmol/mg/min)	Reference
HepG-2	*A. nidulans*	6.3 ± 0.6	4.7 ± 0.9	–	This study
MCF-7	*A. nidulans*	5.6 ± 0.7	4.6 ± 0.9	–	
DLD1	*A. nidulans*	6.4 ± 0.4	3.8 ± 0.7	–	
HT29	*A. nidulans*	9.5 ± 0.6	6.4 ± 0.8	–	
LS174T	*A. nidulans*	12.5 ± 0.8	9.8 ± 0.9	–	
HCT8	*A. nidulans*	12.5 ± 0.9	10.6 ± 0.5	–	
HepG-2	*E. dentata*	20.5 ± 0.7	5.6 ± 0.8	–	Data under publication
MCF-7	*E. dentata*	11.8 ± 0.9	5.4 ± 0.6	–	
HepG-2	*A. fumigatus*	13.9 ± 3.1	4.4 ± 0.9	16.56 ± 4.2	El-Sayed et al., 2015
MCF-7	*A. fumigatus*	16.6 ± 3.6	5.2 ± 1.0	24.2 5 ± 4.6	
HCT8	*A. fumigatus*	22.1 ± 2.0	3.9 ± 1.0	13.23 ± 3.7	
HCT116	*Mycoplasma* sp	–	–	1.42	Alexandrou et al., 2018
RKO		–	–	1.18	
HT29		–	–	0.89	
MH134*	*M. arginini*	10	–	–	Takaku et al., 1995
MH134*	*M. hominis*	100	–	–	
SKMEL28*	*P. plecoglossicida*	100	–	–	Cheng et al., 2014
G361*		120	–	–	
HepG-2*	*E. faecium*	–	–	1950	Kaur et al., 2015

* The enzyme activity was expressed by the concentration of protein (ng) per ml.

expression [46,13], the downregulation of ASS was mainly due to its epigenetic transcriptional modifications, especially by CpG hypermethylation of promotors [10]. ADI is a nonhuman enzyme, so this enzyme has been derived from microbial sources for various preclinical studies. ADI from *M. arginini* was the firstly recognized enzyme with potential anticancer activity against various L-arginine auxotrophic cancers, however, the structural instability, short biological half-life time, conformational frequency of surface epitopes, enzyme antigenicity, are the main challenges that limits its further implementation. In an endeavor to overcome these challenges, several approaches have been developed; Firstly, chemical modification of ADI *via* polyethylene glycol [16,7] and dextran conjugation [30,23] that greatly improve the pharmacokinetic, catalytic and antigenic properties of ADI. Secondly, screening for novel sources of ADI, especially from thermophilic fungi, *A. fumigatus,* displaying a higher structural stability comparing to bacterial ADIs [23,23], nevertheless, its lower catalytic

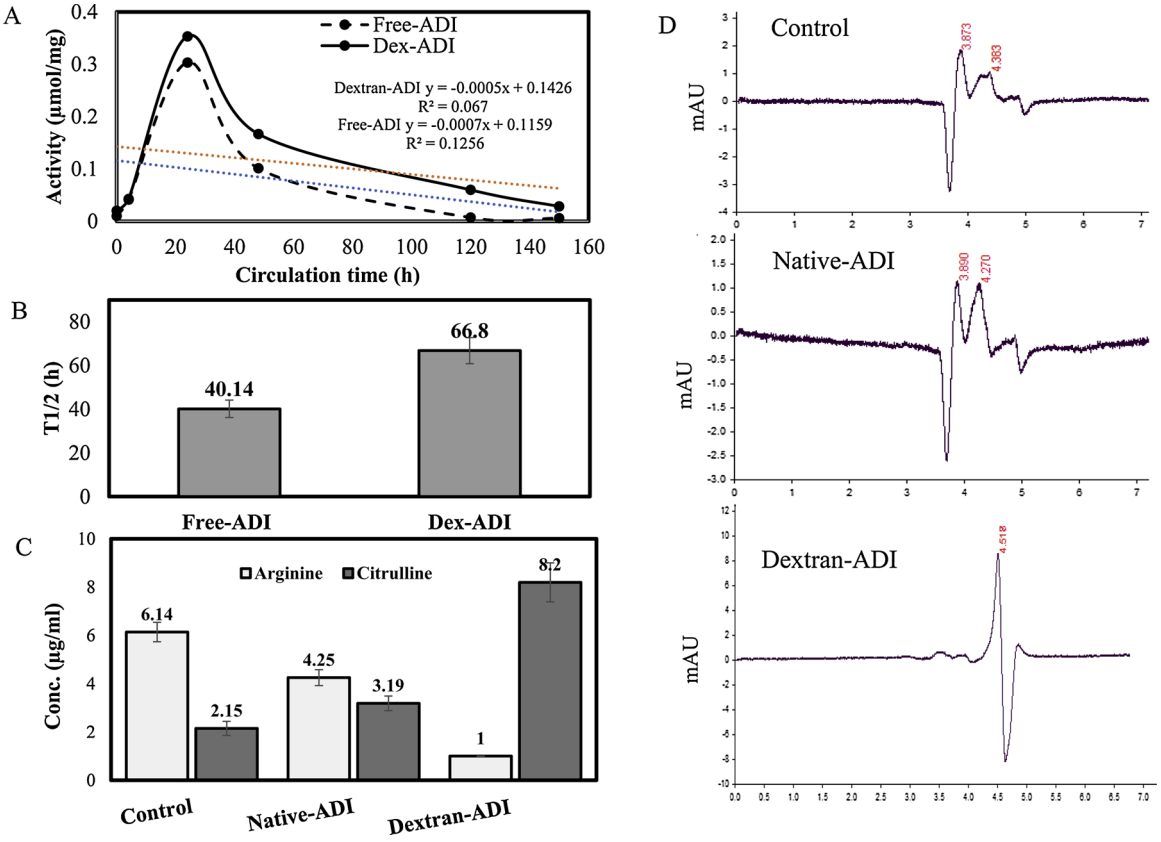

Fig. 6. *In Vivo* pharmacokinetic parameters of the experimental mice in response to single dose of native and Dextran-ADI conjugates. A, the activities of native and Dextran-ADI conjugates were determined intervally up to 150 h circulation time. B, Biological-half time of the native and Dextran-ADI conjugates in mice. C, Serum L-arginine and citrulline concentrations determined by HPLC after 24 h of enzymes circulation. D, HPLC chromatograms of L-arginine and citrulline after 24 h of enzyme circulation. The retention times of L-arginine and citrulline were 3.873 and 4.383, respectively.

Table 8
Biochemical parameters in sera of mice in response to native and Dextran-ADI injection after 4, 24 and 48 hours.

	Control	Native-ADI			Dextran-ADI		
		4	24	48	4	24	48
ALT (U/L)	30 ± 2.0	54 ± 6.3	80 ± 7.8	45 ± 6.2	100 ± 16.4	125 ± 14.6	50 ± 9.0
AST (U/L)	120 ± 15	216 ± 15.3	340 ± 18.9	215 ± 21.2	380 ± 35.5	375 ± 36.8	295 ± 15.0
ALP (U/L)	250 ± 25	324 ± 27.6	314 ± 31.0	270 ± 32.4	393 ± 31.9	639 ± 41	290 ± 14.9
T. protein (g/dl)	4.94 ± 0.9	5.49 ± 0.9	4.54 ± 1.0	5.23 ± 1.2	7.05 ± 1.2	5.53 ± 1.4	5.28 ± 0.9
Albumin (g/dl)	2.91 ± 0.8	1.44 ± 0.2	2.23 ± 0.9	2.57 ± 0.9	2.95 ± 0.3	2.65 ± 0.9	2.33 ± 0.8
Globulin (g/dl)	2.03 ± 0.7	4.05 ± 0.5	2.31 ± 0.8	2.66 ± 0.7	4.1 ± 0.9	2.88 ± 0.6	2.95 ± 0.8
A/G ratio	1.43 ± 0.8	0.36 ± 0.1	0.97 ± 0.1	0.97 ± 0.09	0.72 ± 0.01	0.92 ± 0.2	0.79 ± 0.5
Urea (mg/dl)	31.4 ± 5.2	22.5 ± 4.0	46.5 ± 12.9	33.8 ± 11.0	33 ± 8.0	33.2 ± 8.0	38.5 ± 9.8
Creatinine (mg/dl)	0.65 ± 0.1	0.63 ± 0.1	0.65 ± 0.08	0.47 ± 0.05	0.55 ± 0.03	0.6 ± 0.1	0.44 ± 0.07

efficiency was the main challenge. Thus, we have been motivated to explore ADI with robust thermal stability, higher catalytic efficiency and lower antigenicity from a novel source of thermophilic fungi.

ADI was purified from the mycelia of thermophilic *A. nidulans* by gel-filtration and ion-exchange chromatography [24] with 5.3 purification folds increment. The molecular subunit structure and entire mass of purified *A. nidulans* ADI was 48 and 120 kDa by SDS-PAGE, native-PAGE and gel filtration column, suggesting the homotrimeric identity of this enzyme. The molecular fingerprinting of *A. nidulans* ADI has been determined from the SDS-PAGE in-gel sequencing by LC–MS/MS, annotation with different ADI sequences. The partial peptide sequence of *A. nidulans* ADI displayed 60% similarity with *M. avium* (WP009954974.1), *M. tuberculosis* (NP215517.1) and *Rhodococcus* sp (WP009478910) with 85% coverage. The conserved active sites domains of *A. nidulans* ADI (Glu^{95}-Gly^{96}-Gly^{97}) was identical to *Mycobacterium* sp, *Streptomyces* sp, *Pseudomonas* sp, *Streptococcus* sp, *Clostridium* sp and *Lactobacillus* sp ADI. The upstream and downstream shift on position of these active sites has been shown to be unique for the same enzyme from different sources [6,26]. Four clades of ADIs were committed based on their sequence homology, while *A. nidulans* ADI had a higher proximity to *Mycobacterium* sp clade (IV).

Dextran conjugation has been recognized as a powerful chemical modification technology for various therapeutic proteins [30]. The antigenicity, conformational stability and proteolysis are the challenges that restrain the broad therapeutic applications of non-human enzymes [6] [47,42]. Thus, in an endeavor to maximize the anticancer activity of the purified *A. nidulans* ADI, the enzyme was conjugated with activated dextran [48]. To obtain the maximum conjugation yield and specific activity, different molar ratios of ADI and activated dextran has been tried. The activity of ADI was increased exponentially with the enzyme molar ratio regarding to dextran concentration. The highest enzyme activity was obtained using 0.078 mM of ADI and 20 mM dextran, 77.8% yield and overall increase to subunit molecular mass to ˜ 100 kDa. Consistently, the activity of methionine γ-lyase was slightly reduced upon dextran conjugation [30]. The molar optimization ratio of dextran to ADI is a technical critical issue, since over conjugation of ADI with dextran could have a tackling influence on active sites binding with substrate. Similar paradigm of molar ratio optimizations was conducted for dextran modification of therapeutic enzymes [30,25] and polyethylene glycol [7,21]. It could be hypothesized that dextran-ADI conjugation is mainly due to interaction with ε-amino groups of lysine residues, fairly with surface amid groups, but not interaction with ADI surface thiols. The slight shift on the NH groups from 2359 to 2362 cm and NC groups from 1315 to 1320 cm of ADI upon conjugation with dextran suggest the deprotonation of NH group.

Since, proteolytic cleavage of therapeutic proteins is the major challenge that limits the efficiency of ADI, so, the shielding of surface recognition proteolytic sites of ADI upon dextran conjugation has been evaluated. The resistance of Dextran-ADI conjugates to proteolysis had been increased by 2.5 folds to proteinase K and trypsin compared to native-ADI, suggesting the shielding of more than 50% of the ADI surface proteolytic recognition sites. ADIs were sensitive to proteolysis with proteinase K than trypsin by about 1.5 times, that have been matched with the *in silico* proteolytic map analyses. The putative number of cleavage sites on surface of ADI were found to be 48 and 11 sites for proteinase K and trypsin, respectively, authenticating the sensitivity of ADI to proteinase K than trypsin. Proteinase K is a serine protease of multiple, predominant recognition proteolytic sites that usually target the peptide bonds adjacent to carboxyl groups of aliphatic and aromatic amino acid with blocked α-amino acids [49]. Thus, the higher proteolysis of ADI upon incubation with proteinase K being reasonable, since this enzyme has a non-specific activity, attacking multiple recognition sites on ADI surface. Similar results approving the masking of surface proteolytic recognition sites of *A. fumigatus* ADI [23], *Penicillium chrysogenum* L-arginase [50] and methionine γ-lyase [30] were reported.

The native and Dextran-ADI conjugates have the same catalytic response to reaction temperature, their optimum activities at 37 °C, with visual reduction to their activities by about 3 folds at 30 °C and 45 °C. Obviously, Dextran-ADI conjugates exhibited a higher thermal stability by 2 folds at the tested temperature, ensuring the acquired structural and catalytic stability of ADI upon dextran conjugation [30]. For storage stability (4 °C), the $T_{1/2}$ of Dextran-ADI was increased by 1.6 folds comparing to native ADI with significant decreasing to the K_r values. The native and Dextran-ADI conjugates had the same catalytic response to reaction pH, pH stability and precipitation pH. The highest activity of both ADIs was measured at pH 7.0–8.0, with significant reduction at pH 3.0 and 10.0, ensuring the negligible effect of dextran conjugation on the ionic structure and kinetics of ADI. Both ADIs displayed the same pH stability in response to different pH, with maximum stability at pH 6.5 to 8.5. Taken together, from the reaction pH, precipitation pH and pH stability, it could be deduced that there is obvious effect on the ionic and conformational structure of ADI upon dextran conjugation [50,30,24].

The catalytic and structural identities of ADIs were predicted responsive to various inhibitors and amino acids suicide analogues. Overall, dextran conjugation stabilizes the catalytic and conformational structure of ADI towards various inhibitors. Intriguingly, by demetallizing with EDTA, ADIs loss ˜ 50% of their initial activities, while with addition of KCl (1 mM), their initial activities were completely restored, endorsing their metalloproteineous identity. There was no increasing on ADIs activity upon addition of divalent and trivalent cations, comparing to residual activity of demetallized ADIs, authenticating the dependency of ADI on K^+ ion as cofactor. Similar results have been reported for *A. fumigatus* ADI and *A. nidulans* PAD [17]. The active sites domains of enzymes were predicted from implementation of amino acid suicide analogues; hydroxylamine, iodoacetate, 6-diazo-5-Oxo-norleucine, guanidine thiocyanate, DTNB, MBTH, H_2O_2 and PMSF [6,42,26]. Overall, chemical conjugation with dextran had a slightly stabilizing effect on the conformational and catalytic structure of ADI. The resistance to H_2O_2 oxidation by ADI had increased by two folds upon dextran conjugation, comparing to the native enzyme, suggesting

the steric protection of active sites residues of ADI by dextran residues [51].

The activity of native ADI was reduced by 2 folds in response to MBTH comparing to Dextran-ADI conjugates, confirming the shielding of surface catalytically reactive amino groups. With addition of hydroxylamine, the activity of Dextran-ADI conjugates was not affected, revealing the stabilizing effect of dextran moieties on surface lysine residues [30]. The activity of native ADI was significantly reduced by 9.5 folds comparing to Dextran-ADI conjugates upon DTNB treatment ensuring the limiting of accessibility of L-cysteinyl from DTNB oxidation [26].

The catalytic efficiency of ADI towards various amino acids was slightly changed upon dextran conjugation. The deaminating activities of ADIs were ranged from 10 to 15% towards the tested amino acids, authenticating the stereospecificity of ADIs to deiminate the guanidino amino group of L-arginine with minor affinity to the functional amino groups. The activity of Dextran-ADI to deiminate bovine serum albumin was reduced by 12 folds comparing to native ADI. Noticeably, upon dextran conjugation, the ADI affinity to the tested peptidylarginine residues was slightly reduced comparing to native ADI. This stereo-conformational change of ADI with dextran residues being catalytically affordable, since, citrullination of peptidylarginine residues usually associated with clinical manifestations *via* interaction with P53 proteins and rheumatoid related protein [52–54]. Thus, the catalytic identity of *A. nidulans* ADIs was mainly as deiminase with slight activity as deaminase (arginase) [50]. Kinetically, the native and Dextran-ADI conjugate displayed a significant affinity to deiminate free L-arginine, followed by Boc-Gln-Arg-Arg-MCA with minor deaminating activity. The affinity and catalytic efficiency of ADI to deiminate free L-arginine was increased by 5.3 and 2.4 folds, respectively, upon dextran conjugation, ensuring the improvement on catalytic properties of ADI. Consistent results were reported for glucose oxidase [55], trypsin [48], methionine γ-lyase [56], ADI [23], arginase [50], cystathionine γ-lyase [20] and other proteins [33] upon dextran conjugation.

The antiproliferative activity of ADIs towards six cell lines; breast (MCF-7), liver (HepG-2) and colon cancers (HCT8, HT29, DLD1 and LS174 T) were assessed. Overall, the activity of ADI had been increased by 1.5–1.8 folds against the tested cell lines upon conjugation with dextran, authenticating the positive effect of dextran moieties on improving the conformational structure of ADI. The fluctuation in response to L-arginine starvation by ADIs might be correlated to the expression and activity of ASS in different cell lines [16,57,58]. The resistance of some tumors to extrinsic L-arginine deprivation by ADI could be due to robustness of their ASS expression [59,16]. The complete or partially loss in ASS expression, and consequent arginine auxotrophy have been recorded in more than 90% of the studied tumor cell lines [60,61,15]. Dextran moieties had a dramatic positive effect on improving the kinetic and catalytic properties of ADI against the tested cell lines. Upon dextran conjugation, the *in vivo* ADI activity was increased by about 1.7 folds comparing to the native ADI, ensuring the acquired stability of ADI, due to decreasing on the accessibility of surface proteolytic recognition sites for blood proteases, or decreasing the antigenicity of ADI *via* shielding their surface antigenic epitopes. Coincidently results for various therapeutic enzymes upon dextran conjugation have been reported *in vivo* [27,23,50,21,30]. Interestingly, the native and Dextran-ADI conjugates had no any signs of toxicity *in vivo,* as revealed from the biochemical and hematological parameters. After 24 h, the titer of L-arginine was reduced by 30% and 84% in response to native and Dextran-ADI, respectively, comparing to negative controls. Remarkably, the titer of citrulline in sera was increased by 1.48 and 3.8 folds in response to native and Dextran-ADI treatment, respectively, that coincident with previous reports [7,62]. Thus, upon dextran conjugation, the catalytic activity of ADI to deiminate L-arginine into citrullinate, was increased by about 2.6 folds, comparing to native enzyme, *in vivo*.

In conclusion, *A. nidulans* ADI was purified and molecularly modified *via* covalently conjugation with dextran, and the biochemical properties of both ADI forms were evaluated comparatively. Upon dextran conjugation, the structural and catalytic stability of ADI have been noticeably improved, the affinity towards peptidylarginine was strongly reduced, affording its application with little predicted clinical implication, *in vivo*. Upon dextran conjugation, the anticancer activity and resistance to proteolytic cleavage of ADI *in vitro,* as well as, the biological half-life time *in vivo,* was dramatically increased. The titers of L-arginine in sera was strongly decreased with dramatic accumulation of citrulline by action of Dextran-ADI, comparing to native one. Further studies to explore the mode of action of ADI on cancer cell, especially in combination with various chemotherapeutic agents, have been motivated.

Ethical statement

The animal experiments were conducted according to the guidelines of Institutional Animal Care and Use Committee (IACUC) at Faculty of Medicine, Zagazig University and confirmed to follow NIH guidelines under protocol 15-08-263.

Author agreements

On behalf of all co-authors of manuscript, I hereby confirm the submission of this manuscript to this journal only, and this manuscript has not been submitted to any other journals. All the co-authors are agreed to submit this manuscript to the journal of Enzyme and Microbial Technology.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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