Purification of NADP-Isocitrate dehydrogenase from Red Kidney beans *(Phaseolus vulgaris rogue)*

أستخلاص أنزيم أيسو ستريت دي هايدروجينيس المعتمد على NADPمن الفاصوليا الحمراء وتنقيته

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Abstract

*N*icotinamide adenine dinucleotide phosphate-dependent isocitrate dehydrogenase ($NADP^+$-IDH; EC 1.1.1.42) was extracted from red kidney beans *(Phaseolus vulgaris.)* after the beans were placed into Murashige-Skoog medium and incubated under continuous white light (110 µmol photon m^{-2} s^{-1}), then filtered, centrifuged and the supernatant was used for purification. The enzyme purified using ammonium sulphate precipitation, DEAE-cellulose and Matrex Bio red A (dye-ligand-chromatography) techniques, and exhibits several bands through electrophoresis, with one band corresponds to the IDH activity. Km values for the enzyme was 55.71± 4.56 x 10^{-6}M. The enzyme has an optimum pH at 8.5, and optimum temperature at 30°C. The enzyme can be stable at RT (about 25°C) for 180min, but the activity disappears at 400min. Enzyme activity appears to be independent of divalent metals in deionized water, but the addition of Mg^{+2} and Mn^{+2} by 4.5 and 2-folds respectively. The purified enzyme was injected into white rabbits to raise an antiserum against $NADP^+$-IDH. The specificity of the antiserum was assayed by its ability to decrease the $NADP^+$-IDH activity present in the extract. $NADP^+$-IDH activity decreased when the extract was incubated with increasing volumes of the antiserum obtained.

المستخلص

تم استخلاص أنزيم أيسو ستريت دي هايدروجينيس المعتمد على ($NADP^+$-IDH; EC 1.1.1.42) من الفاصوليا الحمراء الكلية *(Phaseolus vulgaris.)* ووضعت الفاصوليا في وسط موراشكي-سكوج وتم حضنها تحت ضوء أبيض (110 µmol photon m^{-2} s^{-1}) ، ثم تم ترشيح العصير الخام ونبذه واستخدام الراشح لأغراض التنقية ، وتمت تنقية الأنزيم بإستخدام الترسيب بواسطة كبريتات الأمونيوم، وكرموتوغرافيا دي-سليلوز ـ ، ثم ماتركس جيل ريد A ، وأظهر الأنزيم المنقى عدة حزم في الهلام ، كانت واحدة متطابقة مع فعالية الأنزيم ، كما كانت قيمة Kmللأنزيم مساوية إلى . (55.71± 4.56 x 10^{-6}M) ، وكان الرقم الهيدروجيني الأمثل للأنزيم هو 8.5 ، بينما درجة الحرارة المثلى هي 30م ، ويستطيع الأنزيم الثبات في درجة حرارة الغرفة حوالي 25م لمدة 180 دقيقة ، وتختفي الفعالية تماماً بعد 400 دقيقة ، ولا يحتاج الأنزيم فلزات ثنائية التكافؤ عند استخدام الماء عديم الأيونات ، ولكن تتضاعف فعاليته باستخدام Mg^{+2} ، Mn^{+2} ، بمقدار 4.5 و 2 مرة على التوالي ، كما تم حقن الأنزيم المنقى في الأرانب البيض من أجل تكوين المصل المضاد للأنزيم ، وتم تحليل خصوصية المصل المضاد من خلال تحديد قدرته على تقليل فعالية الأنزيم الموجودة في المستخلص . ووجد أن فعالية الأنزيم تقل عندما يتم حضن المستخلص مع أحجام متزايدة من المصل المضاد ، وفي الوقت نفسه التي تظهر إحجام متساوية من المصل الابتدائي تأثير قليل على فعالية الأنزيم .

Introduction

Isocitrate dehydrogenase (IDH) is an enzyme which participates in the tricarboxylic acid cycle. It catalyzes the third step of the cycle: the oxidative decarboxylation of isocitrate, producing alpha-ketoglutarate (α-ketoglutarate) and CO_2 while converting NAD^+ to NADH. This is a two-step process, which involves oxidation of isocitrate to oxalosuccinate, followed by the decarboxylation of the beta-carboxyl group to the ketone, forming alpha-ketoglutarate [1, 2]. According to the specificity for the electron acceptor, two enzymes with IDH activity are known, NAD-IDH (EC 1.1.1.41) and NADP-IDH (EC 1.1.1.42) [1].

Another isoform of the enzyme catalyzes a reaction unrelated to the citric acid cycle, and involved in the supply of 2-oxoglutarate for ammonia assimilation and glutamate synthesis in higher plants through the glutamine synthetase/glutamate synthase cycle and uses $NADP^+$ as a cofactor instead of NAD^+[3,4,5].

Since the discovery of tricarboxylic acid cycle, NADP-IDH has been purified from different organisms. In photosynthetic organisms NADP-IDH has been detected in the cytosol, chloroplasts, mitochondria, and peroxisomes. Cytosolic NADP-IDH has been purified from higher plants [1,2] and eukaryotic algae [2], and its cDNA has been cloned from alfalfa [3], soybean [4], potato [5], and tobacco [6]. NADP-IDH activity has also been detected in peroxisomes from spinach leaves [6].

In alfalfa, soybean, potato, and tobacco, other higher plants and eukaryotic algae, the enzyme appears to be a dimer [4, 5, 6, 7]. The enzyme has been purified from several non-photosynthetic eukaryotes such as fungi and animals [8,9], in which it appears to be a 300-kD octamer. The NAD-IDH from yeast is activated by AMP and citrate, whereas the animal enzyme is activated by ADP and citrate [10]. In yeast and animals, the enzyme is composed of two (yeast) or more (animals) different subunits [2,10].

To our knowledge, no NAD-IDH from photosynthetic organisms has yet been purified to homogeneity, mainly because of the low stability of the enzyme [10, 11]. However, partial purifications have been reported from pea [12], potato [5], and the eukaryotic microalga *Chlamydomonas reinhardtii* [2, 3], although it is an allosteric enzyme that exhibits sigmoidal kinetics with respect to isocitrate [11, 12]. The regulatory importance of NAD-IDH in photosynthetic organisms is still under debate.

The research is aiming to purify NADP-IDH from red kidney beans *(Phaseolus vulgaris rogue)* and to study its characteristics.

Materials and Methods

Materials

Raw Red kidney beans obtained from local markets. Metabolites and standard proteins were purchased from Sigma. DEAE-cellulose and Matrex Gel Red A columns were from Pharmacia Biotech. Chemicals for electrophoresis were purchased from Bio-Rad. All other chemicals were supplied by Merck (Darmstadt, Germany). Molecular weights standards were supplied by Sigma.

Preparation of the beans

Red kidney beans were soaked for 24 h in water and sterilized with 10% sodium hypochlorite then placed onto Murashige-Skoog medium [13] and incubated under continuous white light (110umol photon m-2s-1) overnight. The beans were homogenized with (1: 1 w:v) 0.1 M Tris-HCl buffer, pH 8.5, containing 0.1 % 2-mercaptoethanol and 1.0 mM EDTA by a mortar and pestle on ice. Glass beads 50nm were added. The ratio of plant material to buffer to glass beads was 1.0:4.0:0.5. Glycerol was added to the crude extract to a final concentration of 20%. All the extraction and purification steps were carried out at 4ºC.

Purification Methods

Purification steps followed the procedures of Martínez-Rivas *et al* [2].

The crude extract was filtered and strained through four layers of cheese cloth and centrifuged at 3,000g for 20min. The pellet was discarded and saturated ammonium sulphate was added to the supernatant to a saturation of 0-35% (209g/liter of solution) with stirring. The 35% preparation was centrifuged (as before) for 20min, then the pellet was re-suspended in a minimal volume of the above buffer (0-35% fraction). Ammonium sulphate was added (319g/liter of solution) to the 35% saturated supernatant to make 80% saturated preparation which centrifuged as above. The pellet re-suspended in a minimal volume of the above buffer (35-80% Fraction) and desalted with dialyzing tubes then was applied to DEAE-column (1.5 x 10 cm) which had been equilibrated with the above buffer. The column was washed with this buffer until the eluant was free from protein, and the enzyme was eluted with 0-1mM sodium chloride.

DEAE-cellulose peaked fractions were pooled together and applied to Matrex Gel Red-A (dye-ligand-chromatography) column (1.5 x 10 cm). Equilibrated with the above buffer. After the column was washed with the buffer, the proteins were eluted by a linear gradient of 0 to 1mM sodium chloride in the above buffer. The enzyme was eluted at approximately 0.2mM sodium chloride. Glycerol 20% was added to the buffer used in columns to stabilize the enzyme.

Enzyme Assay

The standard assay mixture for the forward reaction contained 0.1 M tris-HCl buffer, pH 8.5, 0.15 mM NADP+, 0.5 mM D,L,-isocitrate and 0.5 mM MgCl2. All the assays were performed at 30ºC in a final volume of 2.5 ml. Initial velocities were determined by measuring the change in absorbance at 340nm uv-vis spectrophotometer. One unit of activity was defined as the amount of enzyme that catalyzed the production of 1umol NADPH min^{-1} [2].

Protein was estimated by the method of Bradford [14] using Bovine serum albumin (BSA) as a standard. Kinetic studies were performed with purified fractions.

Antiserum Production

Polyclonal antibodies against the purified enzyme were raised in male white rabbits (albinos from local market) by immunized them with 200ug of the purified enzyme (the

active pooled fractions from Matrex gel red A) according to the method of Canton *et al* [12].

Forty days later, the animals were boosted again by the same amount. Ten days later, blood was collected from the ear marginal vein. Samples were allowed to clot for 30 min, and the serum was recovered by centrifugation at 3,000g for 5 min at 4°C. The final preparation was divided into aliquots and stored at -20°C until use.

The enzyme was incubated with increasing volumes of the specific antiserum or with a preimmune rabbit serum for 4 h. The immunocomplexes formed were recovered by centrifugation at 5,000*g* for 5 min at 4°C, and enzyme activity was determined in the supernatant as in Methods.

Enzyme Kinetics

A kinetic analysis was done on the purified enzyme following Matrex Gel Red A chromatography. Determination of K_m values for the enzyme was carried out in 0.1mM tris-HCl buffer, pH 8.4, and 5 mM $MgCl_2$ at different concentrations [2].

Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli [15] using 12% polyacrylamide gel. Proteins were located in the gel by staining with 0.1% (w/v) Coomassie brilliant blue R-250 in 25% (v/v) ethanol and 10% (v/v) acetic acid [16]. NADP-IDH activity was located by running a separate lane with pooled active fractions from Matrex gel red A which was cut out from the gel before staining. The lane is cut horizontally into 0.25mm pieces and submerged in 0.1M tris-HCl buffer, pH 8.5 and assayed for activity as in Methods.

Estimation of native molecular weight

The molecular mass of NADP-IDH was estimated by SDS-electrophoresis. Protein markers of known size: Ferritin from equine spleen (480,000), Urease from Jack bean-trimer- (272,000), Catalase from *Aspergillus niger* (240,000), Myosin from rabbit muscles (205,000), B-Galactosidase from *Escherichia coli* (116,000) and Fumerase from porcine heart (48,000) were used as standards.

Results

Purification

The purification of the enzyme is shown in Table (1). The $NADP^+$-dependent IDH was purified to near homogeneity (about 126-fold) with specific activity of 266.7 units.mg^{-1} and a recovery of 21.16%. Starting with the crude extract of red kidney beans, the maximum purification is measured by increase in specific activity following a DEAE and Matrex gel Red A chromatography, respectively. Ammonium sulphate fractionation and DEAE-cellulose did remove most of the contaminating proteins with an increase in the specific activity (Fig.1). The pooled peaked DEAE-cellulose was chromatographed on Matrex Gel Red A and the shape of the peak suggested heterogeneity of the enzyme. The active fractions of the column preserved in -20°, so that little loss of enzyme activity occurred.

Table (1): Purification of $NADP^+$-IDH from red kidney beans

Purification Step	Volume (ml)	Total Activity (units)	Total Protein (mg)	Specific Activity (Units.mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	20	756	143	5.3	100	1
35% to 80% $(NH_4)_2SO_4$	12	531	9.87	53.79	70.23	10.15
DEAE-cellulose	25	410	1.67	245.51	54.23	46.32
Matrex Gel Red A	12	160	0.12	666.7	21.16	125.8

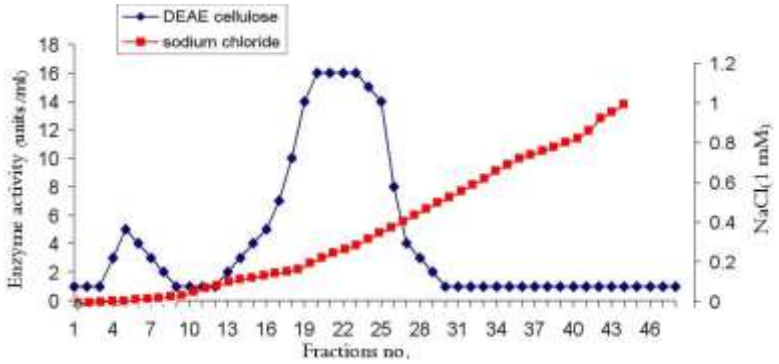

Fig (1): Elution profile of NADP-IDH activity from DEAE-cellulose column (●) with the linear gradient of sodium chloride (0-1mM) (■)

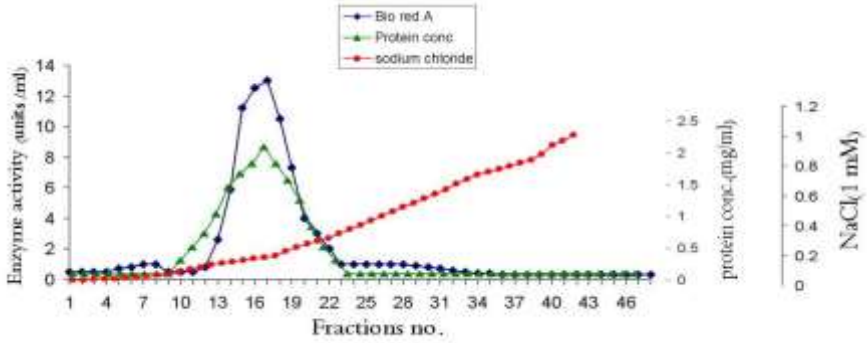

Fig (2): Elution profile of NADP-IDH activity from Matrex gel Red A column (●) with its protein profile (▲) and the linear gradient of sodium chloride (0-1mM) (■)

Optimum pH and Temperature

The enzyme has a maximum activity at pH 8.5 (Fig.3), while the enzyme has an optimum temperature at 30°C that but decreases slowly up to 50°C, but dropped quickly and disappears completely at 60°C. Figure (4)

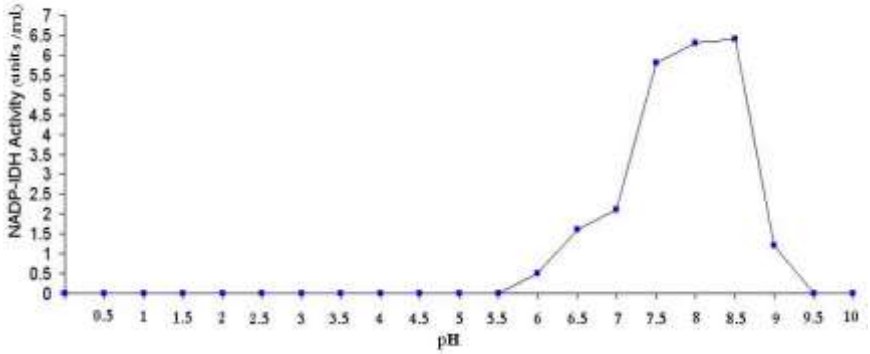

Fig. (3): The effect of pH on the purified NADP-IDH activity from Matrex gel red A

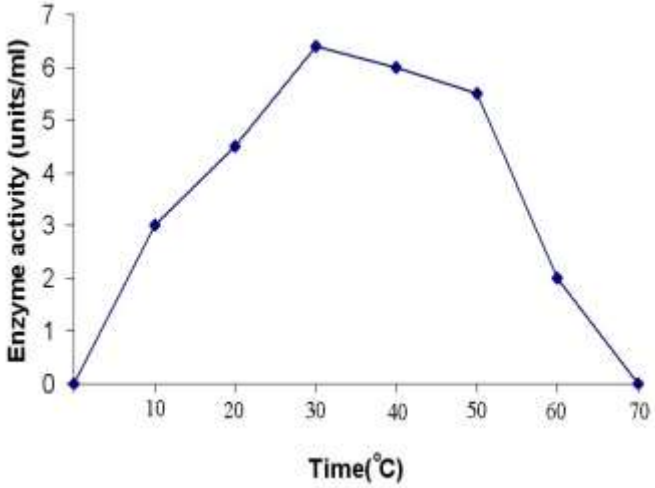

Fig. (4): The effects of temperature on purified NADP-IDH activity from Matrex gel red A

Enzyme Stability

The enzyme initially released from the beans is very stable up 120h at 4°C, then activity dropped slowly until it disappears at 390h. The addition of 20% glycerol stabilized the enzyme substantially (up to 210h) before dropping slowly until all activity disappears at 450h. Sodium chloride accelerates the loss of activity (up to 90h) before dropping fastly until all activity disappears at 300h Figure (5).

The enzyme can be stable at RT (around 25°C) for 6h only, and all activity declines rapidly afterwards until it disappears completely at 6h (results not shown). BSA (1-10%) did not stabilize or activate the enzyme, and it has no effect at all.

All enzyme purification steps were run in 20% glycerol and any higher concentration will interfere with the chromatography steps.

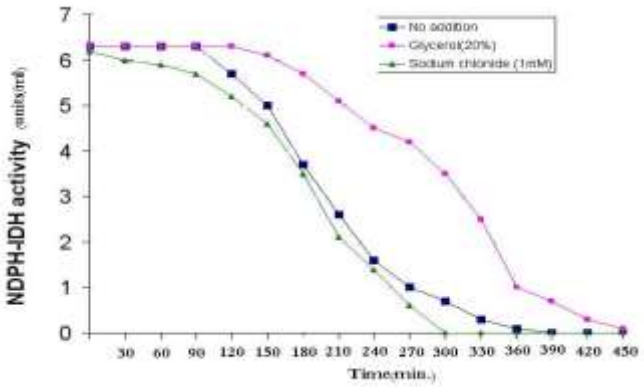

Fig (5): The stability of purified NADP-IDH activity from Matrex gel red A without the addition (■) and with the addition of glycerol (●) and sodium chloride (▲) at 4°C.

Divalent metals effects

The enzyme shows activity in the absence of any divalent metal ions, but Mg+2 and Mn+2 increased that activity by four- and two-folds respectively. Other divalent ions will have inhibitory effect on the enzyme. EDTA addition inhibited the enzyme completely. Table (2).

Table (2): Effect of divalent metal ions on $NADP^+$-IDH activity from the pooled fractions from Matrex gel red A

Ion (5mM in deionized water)	Remaining activity (100%)
None	100
EDTA	0
Manganese	210
Manganese	456
Cadmium	30
Calcium	36
Cupric	26
Nickel	16
Cobalt	35
Mercury	40
Zinc	17

Estimation of Molecular mass

The pooled active fractions from desalted ammonium sulphate fractionation, DEAE-cellulose and Matrex gel red A were electrophoresed on SDS-polyacrylamide gel at pH 8.5. Several bands were extending from top to bottom. More than 10 bands were appeared in Matrex gel red A lane (lane 4), Finger (6), but only the 5^{th} and the 6^{th} band have a strong NADP-IDH activities. These activities were identified by running by running a separate lane with pooled active fractions from Matrex gel red A which was cut out from the gel before staining. The lane is cut horizontally into 0.25mm pieces and submerged in 0.1M tris-HCl buffer, pH 8.5 and assayed for activity as in Methods.

By comparing the positions of the 5^{th} and 6^{th} band with standards used as described in Methods Figure (7), NADP-IDH from red kidney beans is clearly a dimmer with two subunits of 200,000 and 230,000 respectively. The native molecular mass is about 400,000.

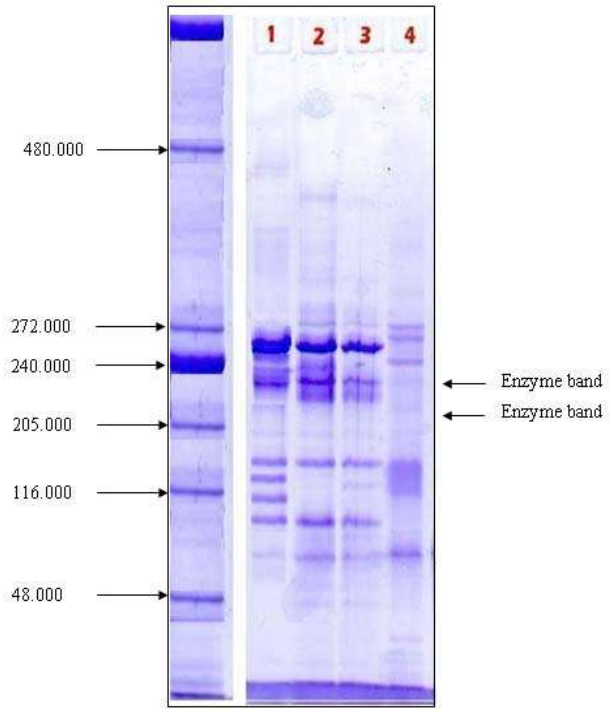

Fig (6): Figure. 8. SDS-polyacrylamide gel with four lanes; Lane 1, A 35-80% desalted ammonium sulphate fraction, Lanes 2 and 3, the pooled peaked fractions from DEAE-cellulose column, Lane 4, the pooled peaked fractions from Matrex Gel Red A. The arrows indicate the bands that show NADP-IDH activity

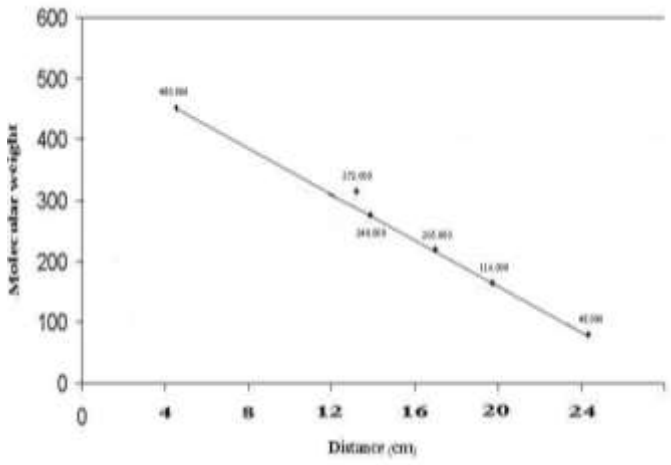

Fig. (7): The mobility of different molecular mass standards to a certain distance that the protein has moved from its point of origin (the beginning of the separating gel)

Enzyme Kinetics

A kinetic analysis was done on the purified enzyme following Matrex Gel Red A chromatography. NADP-IDH showed a standard Michaelus-Menten kinetics for isocitrate and NADP. The Km for NADP-IDH was equal to $55.71 \pm 4.56 \times 10^{-6}$M. Figure (8).

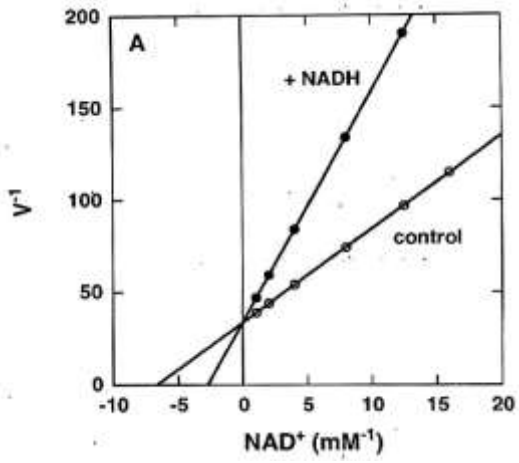

Fig (8): Effect of NADPH on the NADP-IDH activity from red kidney beans. Reaction mixtures were as described in Methods except that the concentration of NADP was varied as indicated in the figure. NADP was added to 0.2 mM final concentration. Initial rates (V^{-1}) are expressed as micromoles of NADP produced by minute

Production of a Polyclonal Antiserum Raised against beans' $NADP^+$-IDH

A purified enzyme was injected into rabbits to raise the antiserum against the enzyme. The specificity of the antiserum was assayed by its ability to decrease the enzyme activity.

The enzyme activity decreased when the enzyme was incubated with increasing volumes of the antiserum obtained, whereas identical volumes of a preimmune antiserum showed little effect on the enzyme activity Figure (9).

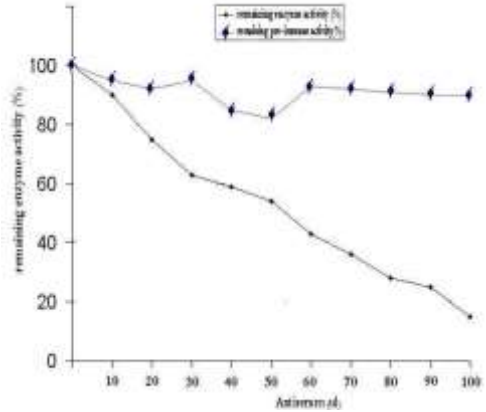

Fig (9): Specificity of the antiserum raised against NADP-IDH activity. The purified enzyme was incubated with increasing volumes of specific antiserum or with a preimmune rabbit serum

Discussion

Purification of red kidney beans enzyme by ammonium sulfate (35% to 80%) saturation, a DEAE-cellulose column, and Matrex Gel Red A column led into a homogeneous preparation of the protein with a recovery of 21.16% and specific activity of (666.7 units.mg^{-1}protein). The shape of the peak from Matrex gel red A Figure (2) suggested that the enzyme may consists of 2-4 subunits. The results from SDS-polyacrylamide gel shows that only two bands of protein (the fifth and the sixth) from Matrex Gel Red A column have strong NADP-IDH activities which suggest that the enzyme is a dimmer with two subunits of 200,000 and 230,000 and a native molecular mass of about 400,000 Figure (6). These results agree with results obtained by previous researchers. Tobacco's NADP-IDH has two forms that have molecular mass of 117,000 and 136,000, respectively [6,7]. Cauliflower's NADP-IDH has a native molecular mass of approximately 100,000 [17] and angiosperm's NADP-IDH is a dimmer of a native molecular mass of 410,000 [10].

NADP-IDH found in several non-photosynthetic eukaryotes such as fungi [1] and animals [10] are octamer or in rare occasions hexamer.

McIntosh and Oliver [18, 19] prefer to use fresh enzyme since subunits can be dissociated by freezing and thawing during enzyme preparation. This unfortunately was not possible during this experiment which may explain the loss of enzymatic activity. Many metal ions can act as chelating agents to the enzyme at concentrations greater than 5mM. This phenomenon was noticed by the present researchers as well by other researchers [2, 6, 11, 17].

The beans' enzyme, unlike most enzymes from plants, can tolerate high degrees of temperature (up to 50°C). Only NADP-IDH obtained from bacteria have the same characteristics [2, 3]. There are many explanation, but most probably that (Red Kidney beans) are rich in Phytohaemoglutinin (PHA) and one of few plants that never give allergic to humans.

Polyclonal antibodies were raised against NADP-IDH which decrease the activity of the enzyme and appears to be a specific to the enzyme.

As shown in Fig.8, the enzyme is a competitive inhibitor (Ki=0.16mM) with respect to NADPH. This was reported for the NADP-IDH purified from pea [20]. This same effect was reported in the enzyme purified from tobacco cells [5] and from higher plant chloroplasts [1]. This inhibition may be an important regulatory mechanism of TCA cycle, but further studies are needed.

Unlike some earlier reports [2, 3, 4], citrate (0.1mM) seems has no effect on the isolated enzyme from red kidney beans. The physiological role of citrate will be investigated in forthcoming paper.

In conclusion, we have described the purification and characterization of $NADP^+$-IDH from red kidney beans. Nevertheless, NADP-IDH role as a key regulatory point in the

carbon flow through the TCA cycle and its participation in the 2-oxoglutarate supply for ammonium assimilation will hopefully be studied in following papers.

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