An Experiment Illustrating the Effect of Saline Stress and Ions on the Malate Dehydrogenase Activity in Vegetal Tissues

ARTICLE *in* BIOCHEMISTRY AND MOLECULAR BIOLOGY EDUCATION · NOVEMBER 2002

Impact Factor: 0.65 · DOI: 10.1002/bmb.2002.494030060129

3 AUTHORS, INCLUDING:

Hugo Virgilio Perales-Vela

Universidad Nacional Autónoma de México

7 PUBLICATIONS **191** CITATIONS

BIOCHEMISTRY AND MOLECULAR BIOLOGY EDUCATION
Vol. 30, No. 6, pp. 384–389, 2002

Laboratory Exercises

An Experiment Illustrating the Effect of Saline Stress and Ions on the Malate Dehydrogenase Activity in Vegetal Tissues*

Received for publication, April 18, 2002, and in revised form, July 12, 2002

Sergio González-Moreno‡, Hugo Perales-Vela, and Martha O. Salcedo Alvarez

From the Laboratorio de Bioquímica, Unidad de Morfofisiología, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Los Rayes Iztacala, Tlalnepantla, CP 54090, México

This paper describes a simple, rapid, and economic protocol to evaluate the effect of saline stress and Na^+, K^+, Ca^{2+}, and Mg^{2+} ions on malate dehydrogenase activity in crude extracts from the leaves and roots of *Vigna radiata*. The main objectives of this work are to illustrate the effect of an environmental stress factor, as well as the *in vitro* effect of some ions on an enzymatic activity, while introducing the students to the use of Michaelis-Menten kinetics and a graphic procedure to calculate kinetics constants.

Keywords: Malate dehydrogenase, saline stress, mung bean, *Vigna radiata*, ions.

The practical exercise proposed here was included within the practical work of a module entitled "Cellular Biology and Biochemistry." This module forms part of the third semester of undergraduate curricula for students majoring in biology.

Salinity stress is known to retard plant growth through its influence on plant metabolism. Some of the affected processes are osmotic adjustment, nutrient uptake, protein and nucleic synthesis, photosynthesis, respiration, several enzyme activities, hormonal balance, and water availability to crop plants [1].

Specific ion toxicity is the primary cause of plant mortality at higher levels of salinity. The osmotic threshold concentrations for injury vary for every salt, and the relative toxicities of specific salts are also different for all crop plants [2]. It has been demonstrated that high concentrations of NaCl, KCl, Na_2SO_4, and K_2SO_4 affect plant growth, metabolism, and photosynthesis of mung bean [3–7].

Crop species with different salt tolerance genotypes, when grown under increasing levels of NaCl salinity, show distinct morphological differences, as well as alterations in behaviors of key enzymes of various metabolic pathways [8–10]. The various isoforms of malate dehydrogenase (L-malate:NAD^+ oxidoreductase; EC 1.1.1.37) catalyze the interconversion of oxaloacetic acid and malate [11]. This malate dehydrogenase (MDH)1 is one of the more active enzymes in peroxisomes, mitochondria, chloroplast, glyoxysomes, and cytoplasm. The isoforms localized in glyoxysomes, peroxisomes, mitochondria, and cytoplasm are NAD^+-dependent whereas the chloroplastic isoform is $NADP^+$-dependent [11]. The kinetic constant values for the mitochondrial MDH are very similar to those of glyoxysomal MDH. K_m for malate is 4 mM, and V_{max} for its oxidation is 5–6 μmol/min. The oxaloacetate K_m is 0.15 mM, and the V_{max} for its reduction is 19 μmol/min. K_m for NADH is 0.11 mM and 0.46 mM for NAD^+ [11]. The oxaloacetate K_m for cytoplasmic MDH from cotyledons of mung bean is 1 mM [12]. Differential expression of MDH isoforms and changes in their activities have been reported in several plant species under abiotic stresses [10–13].

The objectives of this practical exercise are as follows: (i) to evaluate the effect of saline stress (NaCl) on malate dehydrogenase activity in crude extracts from the leaves and roots of mung bean; (ii) to evaluate the effect of Na^+, K^+, Ca^{2+}, and Mg^{2+} *in vitro* on the malate dehydrogenase activity; and (iii) introduce the students to the use of Michaelis-Menten kinetics and the use of a graphic procedure to calculate kinetics constants.

This experiment can be done with three-four students in a group and is designed for two laboratory sessions of 3 h each. The first session involves the establishment of hydroponic cultures, a discussion on the principles of the assay of malate dehydrogenase, and performance of standard assays for protein content and malate dehydrogenase activity using a sample of crude extract prepared by the technical staff. A week later, in the second session, students prepare the crude extracts of roots and leaves from mung bean grown with or without 200 mM NaCl and then they measure total protein in each extract and measure the effect of NaCl *in vivo* and the effect of ions *in vitro* on the malate dehydrogenase activity following the protocol presented here.

Following the completion of the assay, students calculate the velocities of NADH oxidation, calculate K_m and V_{max} for the enzyme, and determine the type of inhibition that occurs with Na^+, K^+, Mg^{2+}, and Ca^{2+} *in vitro*. A

* This work was supported by Programa de Apoyo a Proyectos Institucionales para el mejoramiento de la enseñanza (PAPIME) Project 192016 from the Universidad Nacional Autónoma de México.

‡ To whom correspondence should be addressed: Laboratorio de Bioquímica, Unidad de Morfofisiología, FES Iztacala UNAM, Av. de Los Barrios 1, Los Reyes Iztacala, Tlalnepantla, CP 54090, Estado de México. Tel.: 5255-56231186; Fax: 5255-56231155; E-mail: sergon_9@yahoo.com.

classroom discussion is arranged to discuss the results, and finally, students write a journal-style report.

EXPERIMENTAL PROCEDURES

Plant Material—Seeds of mung bean (*Vigna radiata* L.) were surface-sterilized with 3% sodium hypochlorite solution and then imbibed in water for 24 h. Seedlings were grown in agrolite cultures in plastic boxes with Hoagland nutrient solution [14] with or without 200 mM NaCl. Hoagland solution may be substituted by a solution of commercial fertilizer with adequate proportions of nitrogen, phosphorus, and potassium and micronutrients (0.5 g fertilizer/liter). Seedlings were maintained in a growth chamber for 12–15 days at 25 °C, 70% relative humidity, and a 12-h light/dark cycle (irradiance of 70 μmol·m^{-2}·s^{-1}). Every second day, plants were irrigated with distilled water at field saturation capacity. The total malate dehydrogenase (NAD^+ − MDH) activity was assayed in root and leaf crude extracts, obtained from growing seedlings after 12 or 13 days of saline stress or regular medium exposure.

Crude Extract—1 g of leaves or roots were washed with distilled water and cut into small fragments. The fragments were homogenized with 5 ml of cold homogenization medium (50 mM Tris-HCl, pH 7.5, 10 mM mercaptoethanol, 2% polyvinylpyrrolidone (PVP40), 1 mM phenylmethanesulfonyl fluoride, and 5 mM $MgCl_2$) using a chilled pestle and mortar. The resulting tissue suspension was filtered through eight layers of gauze and centrifuged in a microfuge at 14,000 rpm for 4 min. The supernatant was transferred to a test tube and maintained at 4 °C.

Assay of Malate Dehydrogenase—MDH activity was measured in the direction of NADH-dependent reduction of oxaloacetate. The reaction was followed measuring the decrease in absorbance at 340 nm using the method described by Bergmeyer [15] with some modifications. The difference in absorbance (ΔA_{340}) per unit of time is a measure of malate dehydrogenase activity. A set of eight test tubes were placed in a test tube rack, and solutions of 100 mM Tris-HCl, pH 7.4, 1.25 mM NADH, and 6.6 mM oxaloacetate were added into each tube (Table I).

TABLE I
Malate dehydrogenase assay component additions

Final concentration of oxaloacetate	100 mM Tris-HCl, pH 7.4	1.25 mM NADH	6.6 mM oxaloacetate
M	*ml*	*ml*	*liter*
0	2.75	0.25	0
11	2.75	0.25	5
22	2.74	0.25	10
44	2.73	0.25	20
88	2.71	0.25	40
132	2.69	0.25	60
176	2.67	0.25	80
220	2.65	0.25	100

After the thorough addition of all solution mix by vortexing each tube for 10 s, the content of each tube was transferred to a spectrophotometer cuvette (Quartz), and 25–50 μl of crude extract (20 μg of protein:supernatant) were added to start the reaction. The content was mixed immediately (~5 s), and the cuvette was introduced to the spectrophotometer to start recording. The 340-nm absorbance decrease was monitored for 2 min at room temperature. MDH activity was expressed as μmol of NADH oxidized·min^{-1}·mg of protein^{-1} determined by using the molar extinction coefficient of NADH at 340 nm = 6.2×10^3 liters mol^{-1} cm^{-1}. Protein was measured using the method of Bradford [16]. The results were transformed to obtain the Lineweaver-Burk plot (1/[oxaloacetate] *versus* $1/v_i$ (μmol of NADH oxidized·min^{-1})), and the kinetic parameters were calculated using Enzfitter software [17] and graphic treatment of Eisenthal and Cornish-Bowden [18, 24]. The effect of ions on the total MDH activity was assessed by adding NaCl, KCl, $CaCl_2$, or $MgCl_2$ to a final concentration of 250, 250, 20, and 10 mM, respectively. The volume added was compensated by subtracting the corresponding volume of Tris-HCl used in the assay.

RESULTS AND DISCUSSION

Fig. 1*A* shows the total malate dehydrogenase activity in enzyme preparations from roots and leaves of seedlings of mung bean grown 13 days with or without NaCl in the growing medium. As a result of saline stress, less total MDH activity in roots and leaves was observed. Kumar *et al.* [10] showed a similar effect in total, mitochondrial, and chloroplastic MDH activities of salt-sensitive rice. In roots, total MDH appeared to be more sensitive to saline stress than leaves. When comparing the effects of NaCl *in vivo* on MDH from roots and leaves in a Lineweaver-Burk plot (Fig. 1*B*) a different interception to the ordinate and abscissa was observed only in the leaves of the mung bean. However the K_m and V_{max} values calculated with the Michaelis-Menten equation using the Enzfitter program [17] (Table II) showed that the K_m value for oxaloacetate only changed in total MDH of leaf extract from seedlings grown with 200 mM, whereas the V_{max} was the modified parameter in MDH of roots extracted from seedlings grown under the same conditions. Considering a standard error of ± 10% there are not differences in K_m or V_{max} in total MDH of roots and leaves from seedlings grown with or without saline stress (Table II). Similar results were obtained using the graphic treatment of Eisenthal and Cornish-Bowden [18, 24] (Table II). Some possible factors involved in the resulting de-

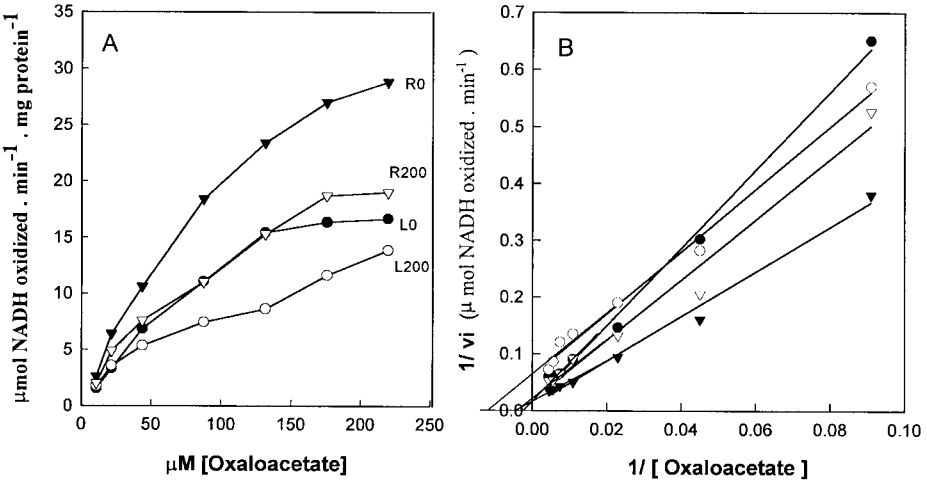

FIG. 1. **Effect of NaCl salinity *in vivo* on MDH activity in crude extract of leaves and roots from seedlings of *V. radiata*.** *A*, substrate *versus* initial velocities. *B*, Lineweaver-Burk plot. ●, leaf control; ○, leaf 200 mM NaCl; ▲, root control; △, root 200 mM NaCl.

crease of MDH activity produced in the plant by saline stress could be a lowered concentration of the enzyme or change of the enzyme form. When the effect of incorporating NaCl, KCl, $CaCl_2$, or $MgCl_2$ in the MDH reaction medium of enzyme extracts from roots and leaves of 13-day seedlings of mung bean grown on non-salinized medium were assayed, a strong inhibition (65–68%) was observed in both tissues at 250 mM NaCl or at 250 mM KCl (Fig. 2, *A* and *C*). Also 10 mM $MgCl_2$ in the reaction medium caused 35 and 50% inhibition in MDH from leaves and roots, respectively, whereas 20 mM $CaCl_2$ caused a 35% inhibition to the MDH in leaves (Fig. 2*A*). Lineweaver-Burk plots of MDH activities from leaves and roots exposed to NaCl or KCl *in vitro* (Fig. 2, *B* and *D*) showed a different interception to the ordinate and abscissa suggesting an uncompetitive inhibition. K_m and V_{max} values calculated with the Michaelis-Menten equation using the Enzfitter program (Table II) showed that 250 mM NaCl and 250 mM KCl *in vitro* produced uncompetitive inhibition, whereas 10 mM $MgCl_2$ *in vitro* produced a reduction in MDH V_{max} on both tissues without alteration on the K_m value and $CaCl_2$ produced a reduction on the K_m and V_{max}. In contrast, the Eisenthal and Cornish-Bowden graphic treatments showed only differences of V_{max} as a consequence of Na^+, K^+, Mg^{2+}, and Ca^{2+} in the reaction medium (Table II) suggesting a non-competitive inhibition in all cases.

Considering the limitations of the Lineweaver-Burk representation, imposed by the crowding of high substrate concentration data points close to the ordinate axis, students can perform an additional analysis following the Eisenthal

TABLE II

Kinetic parameters of MDH in crude extracts of roots and leaves from seedlings of V. radiata grown for 13 days without NaCl, in the presence of 200 mM NaCl, or in the presence of 250 mM NaCl, 250 mM KCl, and 20 mM $CaCl_2$ or 10 mM $MgCl_2$ in the reaction medium

Kinetic constants were calculated with the Michaelis-Menten equation using the Enzfitter program or the graphic treatment of Eisenthal and Cornish-Bowden (in parentheses).

Addition	K_m	V_{max}
	μM	μmol $NAD^+ \cdot min^{-1} \cdot mg\ prot^{-1}$
In vivo		
Leaf None	136 (120)	28 (26)
200 mM NaCl	173 (128)	23 (21)
Root None	154 (160)	50 (52)
200 mM NaCl	155 (130)	33 (36)
In vitro		
Leaf None	196 (172)	41 (32)
250 mM NaCl	729 (140)	24 (9)
250 mM KCl	302 (190)	13 (8)
10 mM $MgCl_2$	151 (160)	19 (21)
20 mM $CaCl_2$	130 (160)	20 (20)
Root None	145 (140)	48 (47)
250 mM NaCl	680 (150)	42 (15)
250 mM KCl	172 (156)	17 (16)
10 mM $MgCl_2$	194 (130)	28 (25)

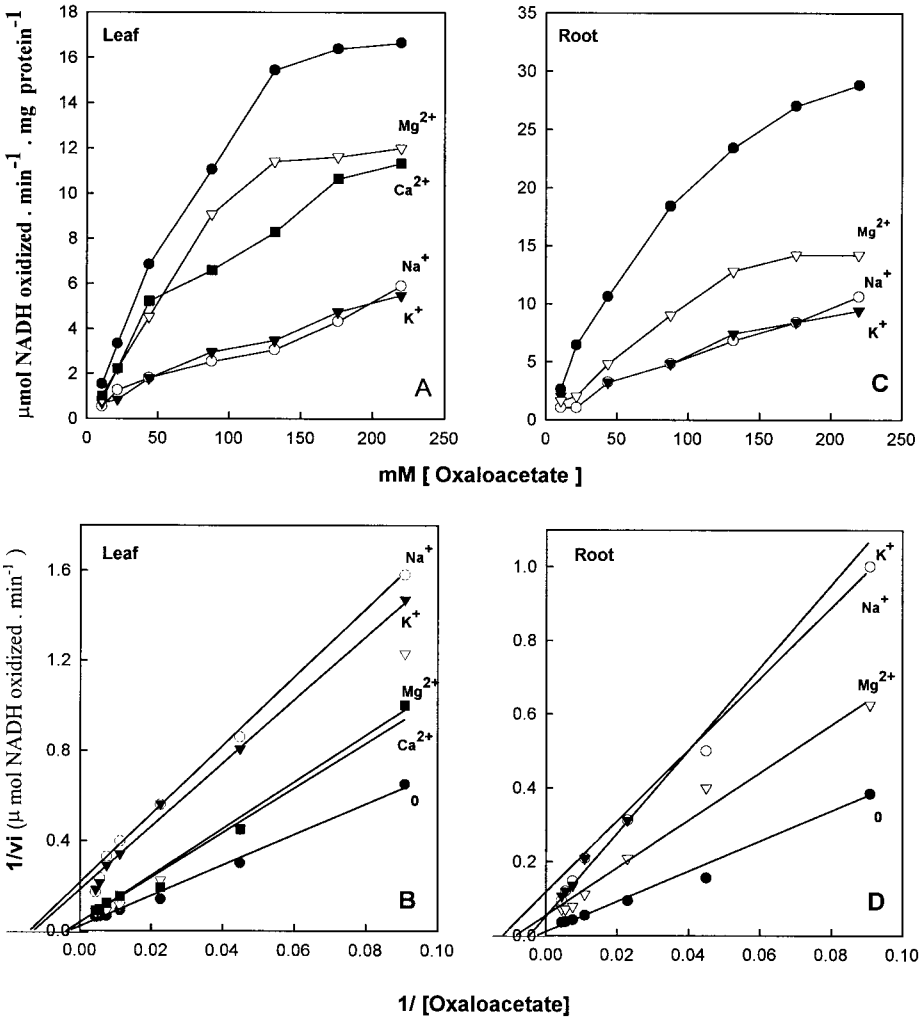

FIG. 2. *A* and *C*, effect of ions *in vitro* on the MDH activity in crude extract of leaves and roots from seedlings of *V. radiata*. *B* and *D*, Lineweaver-Burk plot. ●, control; ○, 250 mM NaCl; ▲, 250 mM KCl; ■, $MgCl_2$; □, 20 mM $CaCl_2$.

and Cornish-Bowden graphic treatments [18, 24].

MDH in plants can be used as a good model to explain the interaction of ions with enzymes and also to study the effect of temperature stresses. This experiment can be extended by isolation and purification of MDH from mitochondria and chloroplast. Good preparations of mitochondria [19] and chloroplast [20] can be obtained from a crude extract of *V. radiata* using differential centrifugation. The same protocol presented here for total MDH activity can be used to determinate kinetic constants and the effects of ions on mitochondrial MDH or chloroplast MDH. Both fractions can be submitted to a native electrophoresis to identity isozyme patterns [21, 22] or can be used to determinate kinetic constants and/or enzyme mechanism for a two-substrate reaction [18, 24–26]. The latter can be performed by determining the kinetic parameters when NADH is held constant, and oxaloacetate concentration is variable and vice versa having oxaloacetate concentration held as constant and varying NADH concentrations. Other plants such as alfalfa, bean, wheat, or sorghum growing in hydroponic culture can be used after a few days of germination.

The main pedagogic benefits of the experiments are that the students would be introduced to the following: (i) the use of hydroponic culture, (ii) the measurement of an enzyme activity, (iii) the use the Michaelis-Menten model and a graphic procedure to calculate kinetic constants, and (iv) explain and interpret the *in vivo* and *in vitro* effect of ions on the enzyme activity. The students are expected to obtain as background information about hydroponic culture [23], determination of enzyme activities [24], quantification of protein concentration [24], the fundamental principles of Michaelis-Menten kinetics, and linear interpretations such as Lineweaver-Burk, Eadie-Hofstee, and Eisenthal and Cornish-Bowden [18, 24–27] and the use of Enzfitter program [17] or a similar program.

Acknowledgment—We thank Dr. Maria Eugenia Torres Márquez, Faculty of Medicine, Universidad Nacional Autónoma de México for correction of the manuscript.

REFERENCES

[1] S. M. Alam, in M. Pessarakli, Ed. (1994) Handbook of Plant and Crop Stress, Marcel Dekker, Inc., New York, pp. 227–246.
[2] G. V. Subbarao, Ch. Johansen, in M. Pessarakli, Ed. (1994) Handbook of Plant and Crop Stress, Marcel Dekker, Inc., New York, pp. 259–279.
[3] I. S. Sheoran, O. P. Garg (1978) Effect of salinity on the activities of RNases, DNase and protease during germination and early seedling growth of mung bean, *Physiol. Plant.* **44,** 171–174.
[4] H. G. Gauch, C. H. Wadleigh (1944) Effect of high salt concentrations on growth of bean plants, *Bot. Gaz.* **105,** 379–387.
[5] M. A. Zayed, L. M. Zeid (1997) Effect of water and salt stresses on growth, chloropyll, mineral ions and organic solutes contents, and enzymes activity in mung bean seedlings, *Biol. Plant.* **40,** 351–356.
[6] A. N. Misra, B. Murmu, P. Sing, M. Misra (1996) Growth and proline accumulation in mung bean seedlings as affected by sodium chloride, *Biol. Plant.* **38,** 531–536.
[7] A. N. Misra, A. Srivastava, R. J. Strasser (2001) Utilization of fast chlorophyll a fluorescence technique in assessing the salt/ion sensitivity of mung bean and Brassica seedlings, *J. Plant. Physiol.* **158,** 1173–1181.
[8] R. S. Dubey, M. Rani (1990) Influence of NaCl salinity on the behaviour of protease, aminopeptidase and carboxypeptidase in rice seedlings in relation to salt tolerance, *Aust. J. Plant Physiol.* **17,** 215–225.
[9] R. Mittal, R. S. Dubey (1991) Behaviour of peroxidases in rice: changes in enzyme activity and isoforms in relation to salt tolerance, *Plant Physiol. Biochem.* **29,** 31–40.
[10] R. G. Kumar, K. Shah, R. S. Dubey (2000) Salinity induced behavioural changes in malate dehydrogenase and glutamate dehydrogenase activities in rice seedlings of different salt tolerance, *Plant Sci.* **156,** 23–34.
[11] C. Gietl (1992) Malate dehydrogenase isoenzymes: Cellular locations and role in the flow of metabolites between the cytoplasm and cell organelles, *Biochem. Biophys. Acta* **1100,** 217–234.
[12] T. Asahi, M. Nishimura (1973) Regulatory function of malate dehydrogenase isoenzymes in the cotyledons of mung bean, *J. Biochem.* (*Tokyo*) **73,** 217–225.
[13] A. H. Kingstonsmith, J. Harbinson, J. Williams, C. H. Foyer (1997) Effect of chilling on carbon assimilation, enzyme activation and photosynthetic electron transport in the absence of photoinhibition in maize leaves, *Plant. Physiol.* **114,** 1039–1046.
[14] P. Hoagland, D. I. Arnon (1938) The water culture method for growing plants without soil, *Plant Physiol.* **17,** 24–26.
[15] H. U. Bergmeyer, in H. U. Bergmeyer, Ed. (1974) Methods of Enzymatic Analysis, Vol. 2, Academic Press, New York, pp. 613–617.
[16] M. A. Bradford (1976) A rapid and sensitive method for the quantitation of microquantities of protein utilizing the principle of dye binding, *Anal. Biochem.* **72,** 248–254.
[17] R. J. Leatherbarrow (1987) *Enzfitter. A non-linear regression data analysis program for the IBM PC* (*and true compatibles*), BIOSOFT, Cambridge, United Kingdom.
[18] R. Eisentahl, A. Cornish-Bowden (1974) The direct plot. A new graphycal procedure for estimating enzyme kinetic parameters, *Biochem. J.* **139,** 715–720.
[19] J. A. F. Vicente, V. M. C. Madeira (2000) The excellence of turnip mitochondrial fractions, *Biochem. Educ.* **28,** 104–106.
[20] R. C. Legood, R. Malkin, in M. F Hipkins, N. R. Baker, Eds. (1986) Photosynthesis Energy Transduction. A Practical Approach, IRL Press, Oxford, United Kingdom, pp. 9–26.
[21] T. C. Moore (1974) Research Experiences in Plant Physiology, Springer-Verlag, New York, pp. 119–129.
[22] G. P. Manchenko (1994) Handbook of Detection of Enzymes on Electrophoretic Gels, CRC Press, Inc., Boca Raton, FL, pp. 39–40.
[23] H. M. Resh (1981) Hydroponic Food Production. A Definitive Guidebook of Soilles Food Growing Methods, Woodbridge Press, Santa Barbara, CA.
[24] R. Eisenthal, M. J. Danson (1992) Enzyme Assay. A Practical Approach, Oxford University Press, New York.
[25] D. Voet, J. G. Voet, Ch. W. Pratt (1999) Fundamentals of Biochemistry, John Wiley & Sons, Inc., New York, pp. 322–349.
[26] D. L. Nelson, M. M. Cox (2000) Principles of Biochemistry, Worth Publishers, New York, pp. 243–292.
[27] I. H. Segel (1975) Enzyme Kinetics. Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, John Wiley & Sons, Inc., New York.