



MEDIA FARMASI

An Indonesian Pharmaceutical Journal

ISSN : 0854-3054 Volume 10 No. 2 Desember 2002

Media pendidikan, penelitian dan informasi ilmiah

Pengasuh :
Pelindung : Rektor USU
Penasehat : Dekan FMIPA USU
Penanggung Jawab :
Ketua Jurusan Farmasi

Ketua Pengarah :
Dr. M. Pandapotan Nasution, MPS., Apt.
Ketua Penyunting :
Dr. Urip Harahap, Apt.

Dewan Penyunting :
Dr. Ginda Haro, Apt.
Dr. Sumadio Hadisahputra, Apt.
Dr. Karsono, Apt.
Drs. Suwandi, MS., Apt.
Drs. Rasmadin Mukhtar, MS., Apt.
Drs. Salim Usman, MSi., Apt.
Drs. Ismail, MSi., Apt.
Dra. Saodah, M.Sc., Apt.
Dra. Masfria, MS., Apt.
Drs. Wiryanto, MS., Apt.

Bendahara :
Dr. Sumadio Hadisahputra, Apt.

Distributor/Promosi :
Dr. Karsono, Apt.
Himpunan Mahasiswa Farmasi

Disain Kulit :
Sumadio Hadisahputra

Diterbitkan oleh :
Jurusan Farmasi
FMIPA USU Medan

Alamat Penerbit/Redaksi :
Jurusan Farmasi FMIPA USU
Jln. Bioteknologi No. 1
Kampus USU, Medan 20155
Telp. 814290 Fax. 814290

AKREDITASI DIRJEN DIKTI NO. 395/DIKTI/KEP/2000

DAFTAR ISI

	Halaman
Pembesaran Gingiva Yang Diinduksi Oleh Obat-obatan, Saidina Hamzah Dalieumunthe	117
Prototipe Pangkalan Data Pengelolaan Administrasi Apotek, Mahyuddin	129
Farmakogenetik Dan Farmakogenomik: Sebuah Pendekatan Baru Terapi Secara Individual, Urip Harahap	142
Uji Stabilitas Tablet Vitamin A: Penetapan Kadar Menggunakan Spektrofotometer Ultra Violet, Effendy De Lux Putra	153
Chromatographic Profile of Isocitrate Lyase During Purification, Erman Munir	162
Pembuatan Membran Alginat Sebagai Sistem Penyampaian Obat Topikal Baru: Povidon Iodum Sebagai Model Obat, Hakim Bangun dan Anayanti Arianto	174
Isolasi Zat Warna Kapsantin Dari Cabe Merah (<i>Capsicum annum</i> L.), Siti Morin Sinaga	183
Analisis Logam Timah (Sn) Dalam Buah Nenas Kemasan Kaleng Dengan Menggunakan Spektrofotometer Serapan Atom, Harry Agusnar	191
Efek Air Perasan Rimpang Jahe Terhadap Pertumbuhan Bakteri <i>Staphylococcus aureus</i> dan <i>Shigella dysenteriae</i> , Siti Morin Sinaga, Rola Yulianti dan Neisy Julita	195

CHROMATOGRAPHIC PROFILE OF ISOCITRATE LYASE DURING PURIFICATION

By:

Erman Munir

Department of Biology, Faculty of Mathematics and Natural Sciences,
The University of Sumatera Utara
Jl. Bioteknologi.No. 1 Kampus USU, Medan 20155

Abstract

Isocitrate lyase, the key enzyme of glyoxylate cycle, was purified to a homogenous protein from the oxalate-producing basidiomycete *Fomitopsis palustris* by several steps of column chromatographies; hydrophobic, weak anion exchange, gel filtration, and strong anion exchange column chromatography, consecutively. Hydrophobic column chromatography successfully separated the enzyme from catalase, which is known as a common contaminant of isocitrate lyase preparation from other sources. The enzyme did not bind to the weak anion exchanger. Then, the purified enzyme with a specific activity of $30.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein was obtained from the strong anion exchanger.

Key words: column chromatography, isocitrate lyase, purification, oxalate-producing basidiomycetes, *Fomitopsis palustris*

Introduction

Isocitrate lyase (threo-D₅-isocitrate glyoxylate lyase, EC 4.1.3.1) catalyzes the reversible cleavage of isocitrate to glyoxylate and succinate. It is known as a key enzyme of glyoxylate cycle, which is commonly found in microorganisms grown on C2 compounds (Kornberg 1966), germinating seeds (Khan, *et al.*, 1992), and larva of nematode (Reiss and Rothstein 1974). A different role of the enzyme has been investigated in many pathogens, such as *Candida tropicalis* (Uchida, *et al.*, 1986), *Coccidioides immitis* (Agy and Paznokaz 1985), *Yersenia pestis* (Hillier and Charnetzky 1981), in riboflavin overproducer *Ashbya gossypii* (Schmidt, *et al.*, 1996), and in rat livers of artificial diabetes of the alloxan treated rats (Povov, *et al.*, 1998). Recently, isocitrate lyase has been discovered as the constitutive enzyme in wood-rotting fungi although they were grown on glucose medium (Munir, *et al.*, 2001a), which contrast sharply with most of the reported cases; little or no activity was detected when microorganisms were grown in the presence of glucose (Schmidt, *et al.*, 1996; O'Connell and Paznokaz 1980; Rua, *et al.*, 1989). Importantly, the enzyme has been found to play an essential role in oxalic acid biosynthesis involving the metabolic

linkage of the tricarboxylic acid and glyoxylate cycles in the wood-rotting fungus *Fomitopsis palustris* (Munir, *et al.*, 2001b),

Although the enzyme has a wide distribution, the highly purified form has been reported only from several species, such as *Phycomyces blakesleeanus* (Rua, *et al.*, 1990), *Bacillus* sp. (Chell, *et al.*, 1978), *Ashbya gossypii* (Schmidt, *et al.*, 1996), *Aspergillus nidulans* (De Lucas, *et al.*, 1997), *Neurospora crassa* (Johanson, *et al.*, 1974), *Pinus densiflora* (Tsukamoto, *et al.*, 1986), *Zea mays* (Khan, *et al.*, 1992), and *Turbatrix aceti* (Reiss and Rothstein 1974). Furthermore, the crystal structure of isocitrate lyase from *Aspergillus nidulans* has been proposed by Britton, *et al.*, (2000). On the basis of the important role of the enzyme in wood-rotting fungi, the enzyme has been recently purified and characterized from for the first time for any of basidiomycetous fungi grown on glucose (Munir, *et al.*, 2002). Then, this paper describes the chromatographic profiles of isocitrate lyase purified from *F. palustris*.

Materials And Methods

Materials

All chemical reagents were of reagent grades. Most chemicals were ordered from Nakalai Tesque. Standard proteins for determination of the molecular mass of the native enzyme were purchased from Sigma Chemicals. The protein assay kit was from Bio-Rad Laboratories. TSK Gel Phenyl-Toyopearl 650 M was obtained from Toyopearl. Protein-Pak DEAE 15HR, HiLoad Superdex 200, and Cosmogel QA and TSK gel G3000SW_{XL} columns were provided by Water, Pharmacia, and Nakalai Tesque, respectively.

Fungus and culture conditions

The prominent oxalic acid-producing basidiomycete *Fomitopsis palustris* was used as the enzyme source. The fungus was grown on potassium phosphate medium (200 ml in 1 liter Erlenmeyer flask) with glucose and peptone as the major carbon and nitrogen source, respectively. The culture was grown statically in darkness at 31°C.

Preparation of cell-free-extracts

Fungal mycelia (from 100 culture flasks) were filtered through cheesecloth and rinsed thoroughly with distilled water and 20 mM potassium phosphate (KPi) buffered containing 1 mM of dithiothreitol (DTT) and ethylenediaminetetraacetate (EDTA). Cell-free extracts were prepared by homogenizing the collected mycelia in a cold mortar in the same way as previously reported (Munir, *et al.*, 2001a). All manipulations were carried out at 4°C, and all buffer solutions contained 1 mM EDTA and 1 mM DTT.

Purification of isocitrate lyase

The enzyme was purified by subjecting the cell-free extracts to the following steps; ammonium sulfate fractionation, hydrophobic, weak anion exchange, gel filtration, and strong anion exchange column chromatography, consecutively. Detailed procedures are described in results and discussion.

Enzyme assays and determination of protein contents

The enzyme activity was assayed spectrophotometrically on the basis of phenylhydrazone formation (at 324 nm), a derivative of glyoxylate produced from isocitrate, by the continuous assay method described by Dixon and Kornberg (1959) with a slight modification. One unit of the enzyme activity is defined as the amount of enzyme that catalyzes formation of 1 μmol of phenylhydrazone per minute under the conditions described. Specific activities were given as units of enzyme activities per mg of protein ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein). Protein contents were determined by the Bradford method using the protein assay kit with bovine serum albumin as a standard (Bradford, 1976).

Molecular mass determinations

The molecular mass of denatured enzyme (subunit) was determined by SDS-PAGE according to the method of Laemmli (1970) in a vertical slab gel instrument using 10-20% (w/v) gradient gel. The subunit molecular mass of the purified enzyme was estimated by use of the standard proteins. The molecular mass of active enzyme (native) was estimated by gel filtration on TSK gel G3000SW_{XL} column (100 mM KPi buffer (pH 7.2), 0.2 ml/min) calibrated with blue dextran, apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

Results And Discussion

Ammonium sulfate fractionation

To the cell-free extracts ($0.4 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein), solid ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) was added slowly to 20% saturation with continuous stirring for one hour. The resulting precipitate not containing the activity was removed by centrifugation, and then additional ammonium sulfate was slowly added to the supernatant to achieve 70% saturation with continuous stirring for three hours. Because at this condition pH dropped from 7.0 to 6.0, NH_4OH solution was added to bring the pH back to 7.0. Importantly, adjustment of pH is necessary to increase the enzyme recovery; 30 % and 90% recovery was obtained when pH was not adjusted and adjusted with NH_4OH solution, respectively. The proteins were collected by

centrifugation at 10,000 rpm for 30 minutes. The pellet thus obtained was dissolved in a small volume of 20 mM KPi buffer (pH 7.2) containing 1 M $(\text{NH}_4)_2\text{SO}_4$ and the undissolved matter was removed by centrifugation ($0.6 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein).

Hydrophobic chromatography

Column was prepared after rinsing the gel with distilled water and starting buffer several times each. The supernatant was applied to TSK gel Phenyl-Toyopearl 650M column (1.5×20 cm) equilibrated with 20 mM KPi buffer (pH 7.2) containing 1.8 M ammonium sulfate. After washing the column, the adsorbed proteins were eluted with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$ (from 1.8 M to 0 M) as shown in Figure 1. The gradient was started at fraction 40 and completed at fraction 130, and isocitrate lyase protein was eluted at $(\text{NH}_4)_2\text{SO}_4$ concentration of 0.5 M, showing with the sharp peak of the

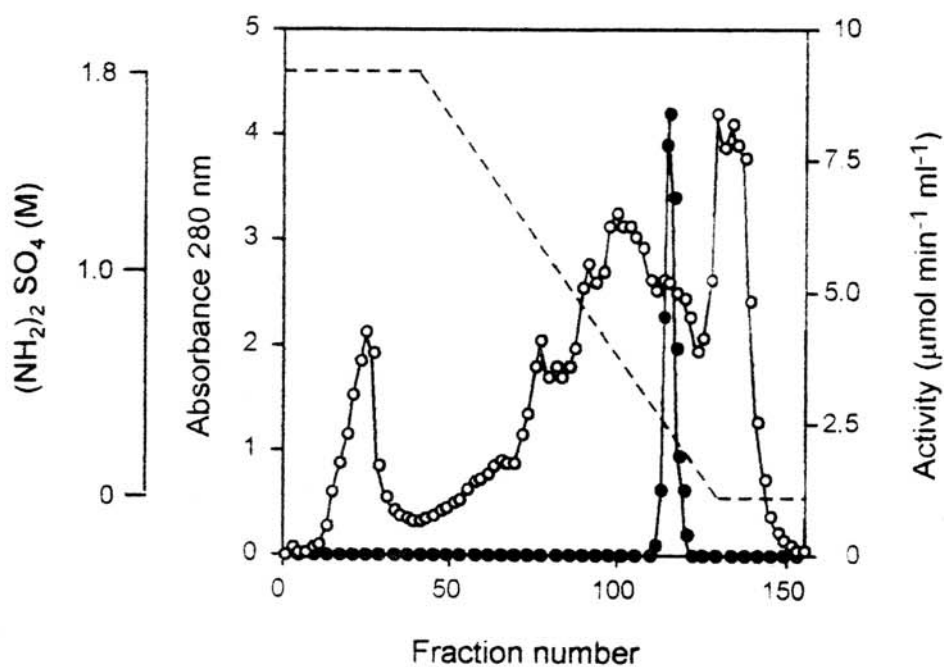


Figure 1. Chromatography of isocitrate lyase on TSK gel Phenyl-Toyopearl 650M. Elution took place with decreasing concentration of $(\text{NH}_4)_2\text{SO}_4$ dissolved in 20 mM KPi buffer (pH 7.2). *Open circle*, absorbance 280 nm; *filled circle*, enzyme activity; *dotted line*, $(\text{NH}_4)_2\text{SO}_4$.

enzyme activity. The results further show that some proteins did not bind to the column but most of them were eluted when salt concentration decreased to about 1.25 M and continued to the end of the gradient. The absorbance spectrum of each activity fraction was monitored to eliminate flavine-containing proteins, which overlapy eluted with isocitrate lyase. Fractions that contain enzyme activity and do not have flavine absorbance (fraction 112 to 118) were combined ($4.5 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein) and dialyzed overnight against 20 mM Tris-HCl buffer (pH 8.0). In addition, hydrophobic chromatography successfully separated the enzyme from catalase, as it was also reported for complete resolution of the *Pinus* isocitrate lyase from catalase (Pinzauti, *et al.*, 1986), which is a frequent contaminant of isocitrate lyase preparation from plant sources (Lamb, *et al.*, 1978).

Weak anion exchange chromatography

The dialyzed enzyme solution was passed through the Protein-Pak DEAE 15HR column (1 × 10 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The adsorbed proteins were eluted with a linear gradient of NaCl (from 0 M to 0.3 M) in 20 mM Tris-HCl buffer (pH 8.0), which was started 10 minutes after enzyme solution was completely loaded with a speed of 1 ml per minute. Because it was found that most of the proteins, but not isocitrate lyase, bound to the DEAE exchanger at several pHs tested, the isocitrate lyase was recovered in the effluents passed through the column; no activity was detected on the effluent fractions. The fractions containing enzyme activity were pooled ($12.7 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein) and concentrated by using concentrators (Centriprep 10, Amicon, Inc.).

In sharp contrast, the isocitrate lyase from other sources such as *Acinetobacter calcoaceticus* (Bahk and Kim 1987), *Colwellima maris* (Watanabe, *et al.*, 2001), and *Neurospora crassa* (Johanson, *et al.*, 1974) were retained by DEAE exchanger. Importantly, although isocitrate lyase did not bind to DEAE exchanger, this step of chromatography significantly separated the enzyme from other proteins, which made of 80% of the injected proteins.

Gel filtration chromatography

Then, the concentrated enzyme was passed through HiLoad Superdex 200 column (1.6 × 60 cm) equilibrated overnight with 20 mM KPi buffer (pH 7.2) containing 0.15 M NaCl. The profile of protein and enzyme activity eluted from HiLoad Superdex 200 column is depicted in Figure 2 showing that the enzyme was located at the first protein peak. Forty-five fractions (to a volume of 45 ml) before the collected fractions were discarded since they did not contain any activity. The fractions containing enzyme activity were combined and dialyzed overnight against 20 mM Tris-HCl buffer (pH

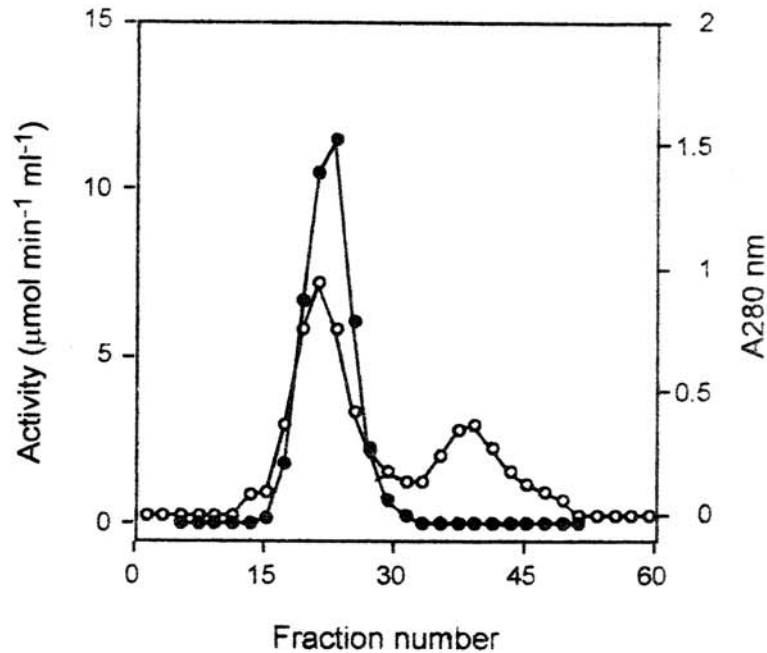


Figure 2. Chromatography of isocitrate lyase on HiLoad Superdex 200. Open circle, absorbance 280 nm; filled circle, enzyme activity.

8.0). The enzyme activity drastically increased to $27.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein or to a purification degree of 68-fold.

In fact, several types of matrix for gel filtration have been used to purify isocitrate lyase. In purification of the enzyme from *A. nidulans*, the purified enzyme was obtained after the enzyme solution was passed through Sephacryl S-300 twice (De Lucas, *et al.*, 1997), whereas Bahk and Kim (1987) and Rua, *et al.*, (1990) used Sephadex during purification of the isocitrate lyase from *P. blakesleeanus* and *A. calcoaceticus*, respectively.

Strong anion exchange chromatography

Eventually, the dialyzate was applied to Cosmogel QA column (0.8×7.5 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The enzyme was eluted with a linear gradient of NaCl (from 0 M to 0.5 M) in 20 mM Tris-HCl buffer (pH 8.0). Results show (Figure 3) that the enzyme tightly bound the column but quickly eluted once the salt strength reached a concentration of about 0.4 M. Furthermore, sharp activity peak (fraction 76 and 77) clearly corresponds with the protein content of related fraction. These two fractions were combined ($30.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein) and filter-sterilized (Millipore $0.22 \mu\text{m}$) and used for enzyme characterization. Within this step of column chromatography the enzyme was purified by 76-fold to a specific activity of $30.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein.

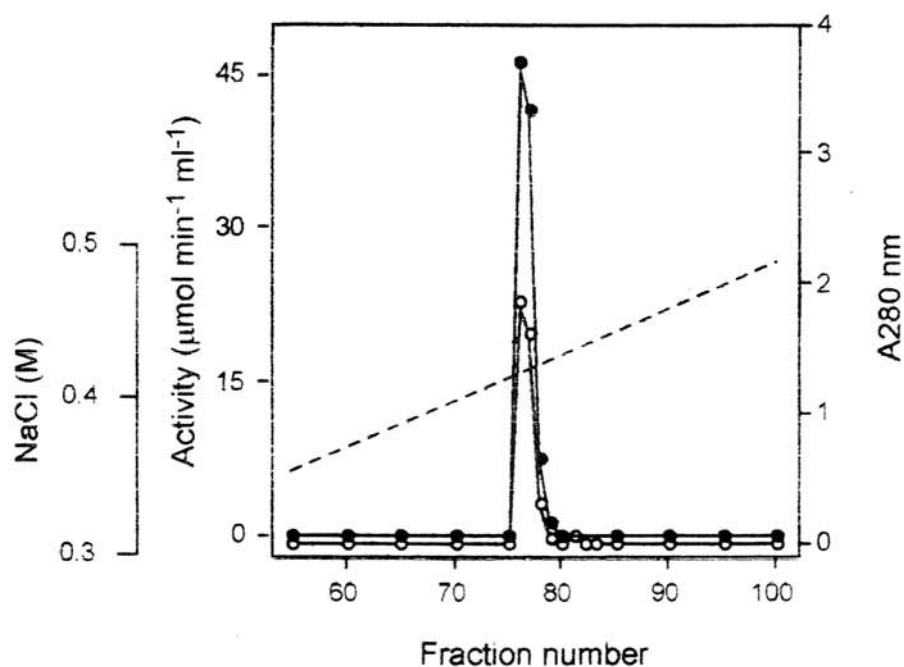


Figure 3. Chromatography of isocitrate lyase on Cosmogel QA. Elution took place with increasing concentration of NaCl dissolved in 20 mM KPi buffer (pH 7.2). *Open circle*, absorbance 280 nm; *filled circle*, enzyme activity; *dotted line*, NaCl.

Molecular mass of isocitrate lyase

Figure 4 shows SDS-PAGE analysis of the enzyme proteins from each purification step, indicating that the purified enzyme gave a homogenous protein band corresponding to a subunit molecular mass of 60 kDa (lane 5). The results also show that the enzyme collected from gel filtration was still contaminated with other proteins (lane 4), which were completely removed by anion exchange chromatography. The isocitrate lyase from *F. palustris* is similar in a monomeric unit size to the enzymes from *Saccharomyces lipolytica* (Matsuoka, *et al.*, 1984) and *Recinus communis* (Roberts and Lord 1981) with the subunit molecular mass of 59 and 62, respectively.

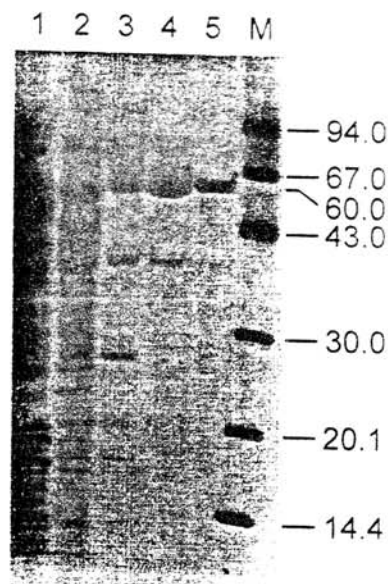


Figure 4. SDS-PAGE analysis of the protein from each column chromatography. *Lane 1*, $(\text{NH}_4)_2 \text{SO}_4$ fractionation (20-70%); *lane 2*, after TSK gel Phenyl-Toyopearl 650M; *lane 3*, after Protein-Pak DEAE 15HR; *lane 4*, HiLoad Superdex 200; *lane 5*, the purified isocitrate lyase (60 kDa) after Cosmogel QA; *lane M*, protein markers with molecular masses indicated on the right (kDa).

Filtration profile of the native enzyme on TSK gel G3000SW_{XL} showed that its molecular mass was estimated to be 186 kDa, as shown in Figure 5. The enzyme was eluted between alcohol dehydrogenase (150 kDa) and β -amylase (200 kDa). Then, it has been proposed that *F. palustris* isocitrate lyase is a trimeric enzyme, which was also confirmed with the electron microscopic investigation (Munir, *et al.*, 2002).

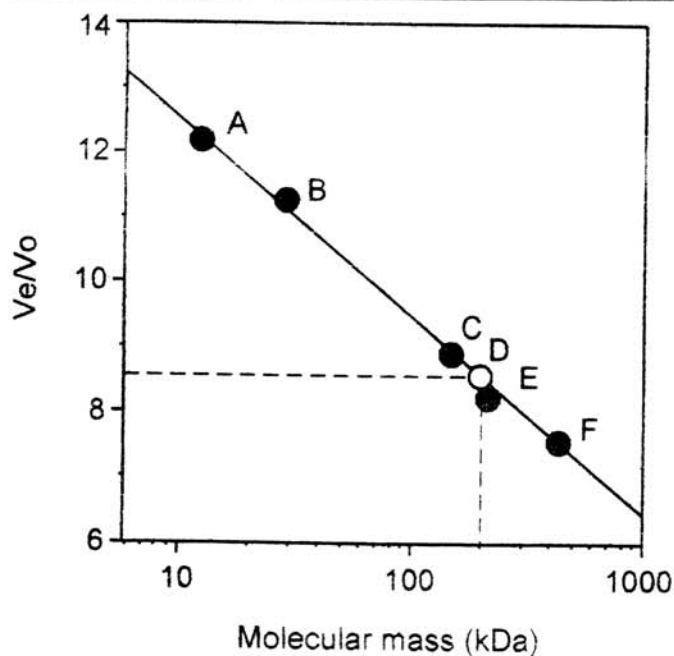


Figure 5. Estimation of the molecular mass of isocitrate lyase by gel filtration chromatography on TSK gel G3000SW_{XL}. A, cytochrome c (12.4 kDa); B, carbonic anhydrase (28 kDa); C, alcohol dehydrogenase (150 kDa); D, purified isocitrate lyase (186 kDa); E, α -amylase (200 kDa); F, apoferritin (443 kDa).

Nevertheless, most of the reported isocitrate lyases were tetrameric enzymes with a subunit molecular mass ranging from 32 to 68 kDa (Vanni, *et al.*, 1990). Thus, it is suggested that the inability of the enzyme to bind to DEAE exchanger was caused by the different structural conformation with the most reported ones. However, further analyses including determination of isoelectric point (pI) and amino acid composition of the enzyme need to be elucidated.

References

- Agy, M.B., and Paznokas, J.L. (1985). Isocitrate lyase and malate synthase activities of *Coccidioides immitis*. *Exp Mycol* **9**: 318–3325.
- Bahk, Y.Y., and Kim, Y.S. (1987). Acidic isocitrate lyase from *Acinetobacter calcoaceticus* grown on malonate. *Korean Biochem. J.* **20**: 191–197.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254.
- Britton, K.L., Langridge, S.J., Baker, P.J., Weeradechapon, K., Sedelnikova, S.E., De Lucas, J.R., Rice, D.W., and Turner, G. (2000). The crystal structure and active site location of isocitrate lyase from the fungus *Aspergillus nidulans*. *Structure* **8**: 349–362.
- Chell, R.M., Sundaram, T.K., and Wilkinson, A.E. (1978) Isolation and characterization of isocitrate lyase from a thermophilic *Bacillus sp.* *Biochem. J.* **173**: 165–177.
- De Lucas, J.R., Amor, C., Diaz, M., Turner, G., and Laborda, F. (1997) Purification and characterization of isocitrate lyase from *Aspergillus nidulans*, a model enzyme to study catabolite inactivation in filamentous fungi. *Mycol. Res.* **101**: 410–414.
- Dixon, G.H., and Kornberg, H.L. (1959) Assay method for the key enzyme of glyoxylate cycle. *Biochem. J.* **72**: 3P.
- Johanson, R.A., Hill, J.M., and McFadden, B.A. (1974). Isocitrate lyase from *Neuspora crassa*. *Biochem. Biophys. Acta.* **364**: 327–340.
- Khan, A.S., Driessche, E.V., Kanarek, L., and Beeckmans, S. (1992). The purification and physicochemical characterization of maize (*Zea mays* L.) isocitrate lyase. *Arch. Biochem. Biophys.* **297**: 9–18.
- Kornberg, H.L. (1966) The role and control of glyoxylate cycle in *Escherichia coli*. *Biochem. J.* **99**: 1–11.
- Laemmli, U.K. (1979) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680–685.
- Lamb, J.E., Riezman, H., and Becker, W.M. (1978). Regulation of glyoxysomal enzymes during germination of cucumber. *Plant Physiol.* **62**: 754–760.

- Matsuoka, M., Himeno, T., and Aiba, S. (1984). Characterization of *Saccharomyces lipolytica* mutants that express temperature-sensitive synthesis of isocitrate lyase. *J. Bacteriol.* **157**: 899-908.
- Munir, E., Yoon, J.J., Tokimatsu, T., Hattori, T., and Shimada, M. (2001a). New role for glyoxylate cycle enzymes in wood-rotting basidiomycetes in relation to oxalic acid biosynthesis. *J. Wood Sci.* **47**: 368-373.
- Munir, E., Yoon, J.J., Tokimatsu, T., Hattori, T., and Shimada, M. (2001b). A physiological role of oxalic acid biosynthesis in the wood-rotting basidiomycete *Fomitopsis palustris*. *Proc. Natl. Acad. Sci. USA.* **98**: 11126-11130.
- Munir, E., Hattori, T., and Shimada, M. (2002). Purification and characterization of isocitrate lyase from the wood-destroying basidiomycete *Fomitopsis palustris* grown on glucose. *Arch. Biochem. Biophys.* **399**: 225-231.
- Nakamura, K., Amano, Y., Nakadate, M. and Kagami, M. (1989) Purification and properties of isocitrate lyase from *Candida brassicae*. *J. Ferment. Bioengin.* **76**: 153-157.
- O'Connell, B.T., and Paznokaz, J.L. (1980). Glyoxylate cycle in *Mucor racemosus*. *J. Bacteriol.* **143**: 416-421.
- Pinzauti, G., Giachetti, E., Camici, G., Manao, G., Cappugi, G., and Vanni, P. (1982). *Arch. Biochem. Biophys.* **244**: 85-93.
- Popov, V.N., Volvenkin, S.V., Eprinsev, A.T., and Igamberdiev, A.U. (1998). Glyoxylate cycle enzymes are present in rat liver peroxysomes of alloxan treated rats. *FEBS Lett.* **440**: 55-58.
- Reiss, U., and Rothstein, M. (1974). Isocitrate lyase from free-living nematode, *Turbatrix aceti*: Purification and properties. *Biochemistry.* **13**: 1796-1800.
- Roberts, L.M., and Lord, J.M. (1981). Synthesis and posttranslational of glyoxysomal isocitrate lyase from castor bean endosperm. *Eur. J. Biochem.* **119**: 43-49.
- Rua, J., Arriaga, D.D., Busto, F. and Soler, J. (1989). Effect of glucose on isocitrate lyase in *Phycomyces blakesleeanus*. *J. Bacteriol.* **171**: 6391-6393.
- Rua, J., Arriaga, D.D., Busto, F., and Soler, J. (1990). Isocitrate lyase from *Phycomyces blakesleeanus*. The role of Mg^{2+} ions, kinetics and evidence for two classes of modified thiol groups. *Biochem. J.* **272**: 359-367.
- Schmidt, G., Stahmann, K.P., Kaesler, B., and Sahm, H. (1996). Correlation of isocitrate lyase activity and riboflavin formation in the riboflavin overproducer *Ashbya gossypii*. *Microbiology.* **142**: 419-426.

- Tsukamoto, C., Ejiri, S., and Katsumata, T. (1986). Purification and some properties of isocitrate lyase from the pollen of *Pinus densiflora* Sieb. Et Zucc. *Agric. Biol. Chem.* **50**: 409-416.
- Vanni, P., Giachetti, E., Pinzauti, G., and McFadden, B.A. (1990). Comparative structure, function and regulation of isocitrate lyase. *Comp. Biochem. Physiol.* **95B**: 431-458.
- Watanabe, S., Takada, Y., and Fukunaga, N. (2001). Purification and characterization of a cold adapted isocitrate lyase and malate synthase from *Colwellia maris*, a psychrophilic bacterium. *Biosci. Biotechnol. Biochem.* **65**: 1095-1103.