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Purification, Identification, and Characterization of Peanut Isocitrate Lyase

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Isocitrate lyase (ICL, EC 4.1.3.1) is commonly present in oil-rich seeds in catalyzing the cleavage of isocitrate to glyoxylate and succinate and plays an essential role in lipid metabolism and gluconeogenesis. When peanut kernels (Tainan 14) were germinated at 30 °C, the cotyledon ICL activities increased substantially in the initial 4 days, and the 4-day-germinated cotyledons were subjected to ICL purification by Tris-HCl buffer extraction, heat treatment at 55 °C for 1 h, $(NH_4)_2SO_4$ fractionation at 25–35% saturation, DEAE-cellulose chromatography, and Sephacryl S-300 gel filtration. A single 64 kDa SDS-PAGE protein band was obtained with 7.7% recovery and 37.5-fold purity. It was identified as ICL by LC-MS/MS analyses and Mascot Search with 494 as the highest Probability Based Mowse Score (PBMS). On the basis of the sequence of the homologous ICL of *Glycine max*, 26% of the peptide sequences of the peanut ICL were identified. During gel filtration, separation of peanut catalase (identified by LC-MS/MS and Mascot Search with 405 as the highest PBMS) from peanut ICL was achieved. The highest measured peanut ICL enzymatic activities were obtained at 45 °C and pH 7.0–7.8, respectively. The enzyme activities were stable (>80%) as stored for 8 h at 30 °C, 15 days at 4 °C, or 60 days at −25 °C. As affected by the supplements in the reactants for activity determinations, ICL activity was not affected by glucose up to 4%, sucrose up to 5%, or ethanol up to 8.33%.

KEYWORDS: Peanut; isocitrate lyase; glyoxylate cycle; LC-MS/MS; catalase

INTRODUCTION

Isocitrate lyase (ICL, EC 4.1.3.1) is one of the essential enzymes of the glyoxylate cycle in the cleavage of isocitrate to succinate and glyoxylate, which is important in gluconeogenesis from the acetyl coenzyme A derived from β-oxidation of the storage triglycerides (*1, 2*). ICL activity is also a control point connecting the balance between fat and carbohydrate metabolisms (*3*). The glyoxylate cycle occurs in organelles called glyoxysomes, distributed in cotyledons or endosperms of dicotyledonous plants and scutellum of monocots. The cycle is also present in microorganisms and some animal embryos (*2, 4*).

ICL has been isolated and studied from various plant sources (*2, 5*). Although the glyoxylate cycle of peanuts has been investigated and peanut ICL and proteins associated with the glyoxysomal fraction increase simultaneously during germination (*6–9*), detailed purification of peanut ICL and its related characterization are still limited. During ICL purification, the intrinsic instability of the enzyme (*4*) and persistent contamination by catalase (*10, 11*) are frequently encountered. In our laboratory, peanut kernels were germinated in preparation of peanut sprouts as vegetable or further applied for biosynthesis of bioactive stilbenoids (*12, 13*). We have noticed that germination of the kernels was driven by ICL activity and also affected by ethanol used for kernel surface disinfection. As reported, when peanut cotyledons are cultured in 2% glucose, the development of ICL and malate synthetase is severely inhibited (*9*). Sucrose is important in seed development and plays a role as an essential agent to drive the progress of the related metabolism (*14, 15*). In the cell culture of cucumber, gene expression of ICL was affected by glucose, fructose, and sucrose (*16*). As related to ethanol, ethanol is produced and consumed by many living organisms. Ethanol treatment triggering a heat shock-like response in soybean has been reported (*17*). Under hypoxic or anoxic conditions, some plants are able to generate energy through ethanolic fermentation (*18*). So far, direct effects of glucose, sucrose, and ethanol on ICL activity have been meagerly investigated. In this study, an improved protocol in the purification of stable peanut ICL was reported. The study was initiated with determination of ICL activity changes during peanut kernel germination. The 4-day-germinated kernel cotyledons were used for ICL purification and LC-MS/MS identification. Separation to eliminate catalase contamination by gel filtration was demonstrated. Investigations on ICL activities as

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affected by temperature, pH, storage, glucose, sucrose, and ethanol were extended.

MATERIALS AND METHODS

Peanut Kernels. Mature peanut pods (*Arachis hypogaea* L. Tainan 14, a Spanish cultivar) were harvested from 2005 and 2006 fall crops. The shelled and graded kernels were deposited in polyethylene (PE) plastic bags and stored at −25 °C for use.

Germination of Peanut Kernels. Peanut kernels in PE bags removed from −25 °C were tempered at the ambient temperature for 30 min prior to opening of the bags. For each batch of germination, 80 sound kernels were placed into a 1000 mL beaker and imbibed with 600 mL of tap water at 30 °C for 6 h in an incubator without light exposure. Then, the kernels were drained and placed onto a plastic net-basket, covered with a wetted paper, and placed into a Polylon cake box (8 in. diameter). The bottom of the box was filled with a layer of tap water to humidify the internal atmosphere. After the box was covered with a lid, the whole box was incubated at 30 °C without light exposure. A series of boxes were respectively incubated for 1−9 days for germination. The 6 h imbibed kernels were designated as 0 days of germination. During germination, the kernels were visually examined daily to discard moldy kernels, followed by rinsing of the kernels with tap water to keep hygiene quality. After germination for each specified interval, cotyledons of the harvested kernels were removed, weighed and distributed into three sublots. From each sublot, a half-amount of cotyledons was subjected to ICL activity determination and the other half was freeze-dried (to determine moisture contents), defatted with cool acetone (*19*), and stored at −25 °C for ICL activity determination.

Enzyme Extraction and ICL and Catalase Activity Determinations. On the basis of extensive preliminary experiments accomplished in peanut ICL extraction and purification, the procedures reported by Ruchti and Widmer (*20*), Polanowski and Obendorf (*21*), and Khan et al. (*22*) were followed with modification. For extraction, each cotyledon sublot was weighed and blended with 4 times (v/w) of buffer (extraction buffer, pH 7.8, 50 mM Tris-HCl buffer, 2.5 mM DTT, 10 mM $MgCl_2$, and 1 mM EDTA) with a blender (Oster, Licuadora Osterizer de 10 Velocidades, Boca Raton, FL) at 4 °C for 1 min. The coarsely blended suspension was further homogenized with a Polytron (PT3000, Kinematica AG, Littau, Switzerland) at 4 °C and 12000 rpm for 4 min. After shaking with an orbital shaker at 4 °C and 75 rpm for 30 min, the solution was centrifuged (35000*g* at 4 °C) (SCR 20B, Hitachi Co., Tokyo, Japan) for 30 min. The middle layer was withdrawn as crude enzyme extract. For extraction of ICL from the defatted peanut cotyledon powder, 0.5 g of the powder was mixed with 5 mL of the extraction buffer and homogenized with a Polytron (PT 3000) at 4 °C (12000 rpm) for 1 min and shaken at 4 °C (75 rpm) for 30 min prior to centrifugation at 4 °C (19000*g*, Sigma Labrozentrifugen 2K15, Osterode, Germany) for 30 min. The supernatant was used as crude enzyme extract for ICL activity determination.

For determination of ICL activity, the procedure reported by Dixon and Kornberg (*23*) and Ruchti and Widmer (*20*) was followed with modification. For each assay, 2.5 mL of the substrate solution (substrate buffer, pH 7.8, 50 mM Tris-HCl buffer containing 5 mM DTT, 3.3 mM phenylhydrazine, 10 mM $MgCl_2$, and 5 mM isocitric acid) was deposited into a test tube and incubated in a water bath at 37 °C for 5 min. Then, 10 μL of the enzyme solution was introduced, thoroughly mixed, and incubated at 37 °C. Absorbance at 324 nm of the reactant after 2 and 10 min was measured spectrophotometrically (U-2001 spectrophotometer, Hitachi Co.). In this study, similar to the results of Khan et al. (*22*), 324 nm absorbance of the reactant was found to increase linearly for at least 15 min. For construction of a reference curve, a series of glyoxylic acid solutions, that is, 0, 10, 20, 40, 60, and 80 μM, were prepared, from which 10 μL aliquots were, respectively, withdrawn to substitute the enzyme solution to combine 2.5 mL of the substrate solutions and reacted at 37 °C for 2 and 10 min prior to absorbance determinations at 324 nm. One ICL activity unit was defined as the release of 1 μmol of glyoxylic acid per minute.

For catalase activity determination, the procedure of Yanik and Donaldson (*11*) was followed. For each determination, 2.5 mL of the substrate solution (pH 7.0, 50 mM phosphate buffer and 13.5 mM H_2O_2) was incubated in a water bath at 30 °C for 5 min. Then, 10 μL of enzyme solution was introduced, thoroughly mixed, and further incubated at 30 °C. Absorbance of the reactant at 240 nm (U-2001 spectrophotometer) was determined after 0.5 and 3 min of incubation. One unit of catalase activity was expressed as 1 absorbance unit increase per minute.

ICL Purification and Protein Quantification. Basically, the procedures reported by Ruchti and Widmer (*20*), Polanowski and Obendorf (*21*), and Khan et al. (*22*) were followed with modifications. The 4-day-germinated cotyledons were extracted with 4 volumes (v/w) of the extraction buffer as described above for the preparation of crude enzyme extracts. As a routine practice in each step of purification, small fractions of the enzyme solutions were sampled for protein quantification, ICL activity determination, and electrophoretic analyses.

For protein quantification, the Bradford method (*24*) in accordance with the manufacturer's procedure (Amerco Co., Solon, OH) was followed. Briefly, the stock protein dye solution was diluted with 3 volumes of deionized water, and a series of standard protein solutions containing 50, 100, and 300 μg/mL of bovine serum albumin (BSA) (Pierce Co., Union City, CA) were prepared and quantified to construct a reference curve. For each determination, 20 μL of the protein sample was well mixed with 1 mL of the prepared protein dye solution in a test tube and incubated at the ambient temperature for 5 min. Absorbance at 595 nm was determined in estimation of protein quantity.

For further purification, the crude enzyme extracts were subjected to heat treatment at 55 °C for 60 min to inactivate some unwanted macromolecules (*22*). After rapid cooling of the heated solution in an ice bath, the solution was centrifuged (35000*g*) at 4 °C for 30 min and the pellet was discarded. The volume of the supernatant was measured and introduced with saturated $(NH_4)_2SO_4$ solution to reach 25% saturation. After the solution had been maintained at 4 °C for 1 h, the solution was centrifuged (35000*g*) at 4 °C for 30 min, and the supernatant was further replenished with saturated $(NH_4)_2SO_4$ solution to reach 35% of saturation. After 1 h at 4 °C, the solution was centrifuged (35000*g*) at 4 °C for 30 min, and the pellet was rapidly dissolved in an appropriate volume of the extraction buffer to maintain the protein concentration at ca. 15 mg/mL. The solution was then deposited into dialysis tubes (Spectra/Por Dialysis Membrane, MW 12000–14000, Spectrum Laboratories, Rancho Dominguez, CA) and dialyzed at 4 °C overnight against elution buffer (5 mM Tris-HCl buffer, pH 7.8, 1 mM DTT, 6 mM $MgCl_2$, and 1 mM EDTA). After the dialyzed solution had been centrifuged (19000*g*) at 4 °C for 30 min (Eppendorf Centrifuge 5415C, Eppendorf, Hamburg, Germany), the volume of supernatant was measured, and small aliquots were sampled for protein quantification and ICL activity determination.

For chromatographic purification, the $(NH_4)_2SO_4$-fractionated enzyme solution was replenished with Triton X-100 to reach 0.2% of concentration (*22*), membrane-filtered, and loaded onto a DEAE-cellulose (Sigma-Aldrich Co., St. Louis, MO) ion exchange column (2.1 × 9.0 cm) for separation. For each run, 2 mL (ca. 15 mg of protein/mL) was loaded and eluted with the elution buffer at 0.8 mL/min and fractionated every 3 mL for absorbance determination at 280 nm and ICL activity determination. The active-enzyme fractions were pooled and concentrated with a UF tube (Amicon Ultracel PL-30 Centrifugal Filter Devices, Millipore Co., Bedford, MA) to a final protein concentration at ca. 15 mg/mL.

For the final purification, 2 mL of the DEAE-cellulose-purified fraction (15 mg/mL) was loaded onto a gel filtration column for chromatography (Hiprep 26/60 Sephacryl S-300 HR, 600 × 26 mm, GE, Uppsala, Sweden) and eluted with the elution buffer and fractionated every 5 min for protein quantification, SDS-PAGE analysis, and enzyme activity determination of ICL and catalase. The ICL and catalase active fractions were, respectively, pooled and subjected to UF concentrating (Amicon Ultracel PL-30 Centrifugal Filter Devices) to reach ca. 15 mg/mL of concentration. The solutions were stored at −25 °C for property characterization.

Gel Electrophoresis of Proteins. The procedure of Laemmli (*25*) was followed for sodium dodecyl (SDS)−polyacrylamide gel electrophoresis (PAGE). The native-PAGE analysis of the proteins was run without treatment with mercaptoethanol and SDS. The electrophoresis

system (Mini-protein 3 system, Bio-Rad Laboratory, Hercules, CA) run with 50 V for stacking and 130 V for separation was conducted. The gels were stained with Coomassie blue and destained with methanol and acetic acid solution. Native-PAGE high molecular weight and SDS-PAGE low molecular weight calibration kits were run concurrently for migration reference.

LC-MS/MS Protein Identification. From the SDS-PAGE gels obtained from analyses of the DEAE-cellulose column purified and concentrated portions, two protein bands with 64 and 56 kDa molecular mass were cut with a scalpel and submitted to the LC-MS center of National Chen Kung University for amino acid sequencing and peptide sequence matching with Mascot Search. The facility and software included LCQ (nano LC) and MS/MS, a C18 reverse phase column (75 μm × 15 cm), and Mascot as search software linked with the NCBI database; the search parameters were set as follows: taxonomy, specified to Viridiplantae (green plants); enzyme, trypsin; modification, carboxymethyl (C, K), deamidation (N, Q), oxidation (M), and Pyro-Glu (D, E). The proteins were identified and expressed by significant hits and protein view.

Characterization of the Purified Peanut ICL. For activity determination of the gel filtration-purified ICL as affected by temperature, 2.5 mL aliquots of the substrate solution were, respectively, incubated at 25, 30, 37, 45, 55, and 65 °C for 5 min prior to introduction of 10 μL of the ICL solution. After further incubation of the reactants for 2 and 10 min, absorbance at 324 nm of the reactants was respectively determined. The relative activity was expressed as percentage in proportional to that determined at 37 °C as 100%.

For activity determination of the gel filtration-purified ICL as affected by pH, a series of the substrate buffers were adjusted with HCl or NaOH to pH 6.5, 7.0, 7.5, 7.8, 8.0, and 8.5 and used for substrate preparation. From each substrate solution, 2.5 mL- aliquots were incubated at 37 °C for 5 min prior to introduction of 10 μL of the ICL solution. After further incubation of the reactants for 2 and 10 min, absorbance at 324 nm of the reactants was respectively determined. The relative activity was expressed as percentage in proportion to that determined at pH 7.8 as 100%.

For thermal stability assessment of the gel filtration-purified ICL, 50 μL aliquots of the ICL solution in 1.5 mL Eppendorf tubes were respectively heated in a series of water baths set at 25, 30, 37, 45, 55, and 65 °C for 15 min. Meanwhile, 2.5 mL aliquots of the substrate buffer solutions were incubated at 37 °C for 5 min to get ready for introduction of 10 μL of the heat-treated ICL solutions. After further incubation of the reactants for 2 and 10 min, absorbance at 324 nm of the reactants was respectively determined. The relative activity was expressed as percentage in proportion to that determined at 37 °C as 100%.

For storage stability assessment, 50 μL aliquots of the gel filtration-purified ICL solution in a series of 1.5 mL Eppendorf tubes were mixed with equal volumes of glycerol and stored at −25 °C. During storage, the ICL activities were repeatedly determined. For ambient and refrigeration storage stability assessment, 50 μL aliquots of the ICL solution in a series of 1.5 mL Eppendorf tubes were respectively stored at 30 and 4 °C. During storage, the ICL activities were repeatedly determined as described above. The remaining ICL activities after storage were determined and expressed as percentages in proportion to the original activity subjected to storage.

For determination of the gel filtration-purified peanut ICL activity as affected by glucose and sucrose, a series of 2.5 mL substrate buffers were incubated at 37 °C for 4.5 min and respectively replenished with 0.5 mL of the buffer solutions containing 0, 1.5, 3, 6, 12, and 24% of glucose (Sigma-Aldrich Co.) or containing 0, 3.75, 7.5, 15, 30, and 45% of sucrose (Sigma-Aldrich Co.). The glucose and sucrose concentrations of the combined solutions were equivalent to 0, 0.25, 0.5, 1.0, 2.0, and 4.0% for glucose and 0, 0.63, 1.25, 2.5, 5, and 7.5% for sucrose, respectively. After 0.5 min, 10 μL of the ICL solution was respectively introduced. After further incubation of the reactants for 2 and 10 min, absorbance at 324 nm of the reactants was respectively determined. The determined ICL activities as affected by glucose and sucrose were expressed as percentages in proportion to that determined without supplementation as 100%.

Table 1. Moisture Contents and Isocitrate Lyase (ICL) Activities of the Peanut Cotyledons during Germination at 30 °C for 9 Days

time, days	moisture content[a] (%, wet basis)	ICL activity[a] (units/mL, fresh peanut)	ICL activity[a] (units/mL, defatted powder)
0	33.4 ± 1.3h	0e	0f
1	35.1 ± 0.7h	0.1 ± 0.1e	0.1 ± 0f
2	41.4 ± 0.5g	0.8 ± 0.2d	2.4 ± 0.1e
3	44.4 ± 0.1f	1.6 ± 0.5bc	3.7 ± 0.1d
4	49.7 ± 0.3e	2.6 ± 0.5a	4.7 ± 0.1a
5	54.6 ± 1.3d	2.5 ± 0.5a	4.7 ± 0.1a
6	58.4 ± 1.4c	2.7 ± 0.3a	4.1 ± 0.2c
7	67.7 ± 1.9b	2.3 ± 0.5ab	4.4 ± 0.2b
8	67.6 ± 0.8b	1.5 ± 0.7c	4.1 ± 0.1c
9	72.7 ± 0.8a	1.0 ± 0.5cd	4.0 ± 0.1c

[a] Mean of determinations ± SD (n = 3); data bearing different letters in the same column are significantly different (p < 0.05).

For determination of the gel filtration-purified peanut ICL activity as affected by ethanol, a series of 2.5 mL substrate buffers were incubated at 37 °C for 4.5 min and respectively replenished with 0.5 mL of the substrate solutions containing 0, 1.56, 3.13, 6.25, 12.5, 25, 50, and 95% of ethanol. The ethanol concentrations of the combined solutions were equivalent to 0, 0.26, 0.52, 1.04, 2.08, 4.17, 8.33, and 16.67%, respectively. After 0.5 min, 10 μL of the purified peanut ICL solution was respectively introduced. After further incubation of the reactants for 2 and 10 min, absorbance at 324 nm of the reactants was respectively determined. The determined ICL activities as affected by ethanol were expressed as percentages in proportion to that determined without supplementation as 100%.

Statistics. At least triplicate experiments were conducted in this study. Means of determination with standard deviation are reported. For comparison, the data were applied for ANOVA for variance and significance analysis.

RESULTS AND DISCUSSION

When peanut kernels were imbibed with water at 30 °C for 6 h and incubated at the same temperature for 9 days, moisture contents of the kernels increased steadily with time of incubation (**Table 1**). The cotyledon ICL activities, expressed either on fresh weight or defatted powder basis, both increased substantially in the initial 4 days, then leveled off for an additional 1−3 days, and then decreased thereafter. This activity pattern was in agreement with some oil-rich seeds in which ICL appears in the oil-storing tissues after seed imbibition, increases up to a climax in a few days, and then declines thereafter (*1, 26*). In this study, ICL was not detected in the cotyledons on day 0 when peanut kernels were imbibed with water for 6 h (**Table 1**). Longo (*8*) and Allfrey and Northcote (*3*) reported that peanut ICL is de novo synthesized after the onset of germination. Observations with cucumber seeds during germination indicate that increases in ICL activity are dependent upon increases of the translatable ICL mRNA (*27*). In this study, to prepare peanut ICL source material for ICL purification, the 4-day-germinated cotyledons were subjected to buffer extraction and followed steps of ICL purification.

When the 4-day-germinated cotyledons were subjected to extraction, heat treatment at 55 °C for 1 h, 25–35% saturated $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography, and Sephacryl S-300 gel filtration, the related measures are shown in **Table 2**. Their corresponding SDS- and native-PAGE protein patterns are shown in **Figure 1**. When the crude enzyme extract was heated at 55 °C for 1 h, the ICL recovery and purification were 95.6% and 1.3-fold, respectively. As shown by their PAGE protein patterns, the proteins representing ICL (shown with the upper arrows) were not affected, but some macromolecular

Table 2. Purification Table for Isocitrate Lyase from Peanut Cotyledons

purification step	total activity (units)	total protein (mg)	specific activity (units/mg)	recovery (%)	purification (fold)
crude extraction	588	4912	0.12	100	1.0
heat treatment	564	3721	0.15	95.9	1.3
ammonium sulfate fractionation	216	180	1.20	36.7	10.1
DEAE-cellulose chromatography	95	26	3.65	16.2	30.4
Sephacryl S-300 gel filtration	45	10	4.50	7.7	37.5

proteins were altered. This was in agreement with the observation reported by Khan et al. (*22*) in the purification of maize ICL.

In the following 25–35% saturated $(NH_4)_2SO_4$ fractionation, the ICL purification was increased up to 10-fold and its enzyme recovery was 36.7% (**Table 2**). In this step, most unwanted proteins were removed (the lower molecular weight molecules) as shown both in SDS- and native-PAGE analyses (**Figure 1**). When the fractionated fractions were subjected to DEAE-cellulose column chromatography, as again shown by their SDS- and native-PAGE protein patterns (**Figure 1**), two distinct bands with 64 and 56 kDa molecular masses (shown with arrows) were obtained. For identification, the bands were cut and subjected to LC-MS/MS analyses of peptide amino acid sequences and Mascot Search with NCBI nr 20061201 (4196452 sequences; 1444328266 residues). As shown in **Figure 2A**, five significant hits with Probability Based Mowse Scores (PBMS) up to 494 were matched with the green plant ICLs. This provides sufficient evidence to identify the single band with a molecular mass of 64 kDa as peanut ICL. In protein view for comparison, based on the sequence of the homologous ICL of *Glycine max*, 26% of the peptide sequences of the peanut ICL were identified. As reported by Polanowski and Obendorf (*21*) in testing against soybean ICL-1 antibodies, the protein extracts from the germinating peanut seeds exhibit immunogenic cross-reactivity and bear partial similarity to soybean ICL. A 64 kDa ICL was also extracted and purified from maize (*22*). As shown in **Figure 2B** for researching the 56 kDa band, 12 significant hits with PBMS up to 405 were matched with green plant catalases. This also provides sufficient evidence to identify the single band with a 56 kDa molecular mass as peanut catalase. In comparison, on the basis of the sequence of the homologous catalase of *Cucurbita pepo*, 21% of the peptide sequences of the peanut ICL were identified.

In this study, two bands representing peanut ICL and catalase after DEAE-cellulose chromatography were detected in SDS- and native-PAGE analyses (shown with arrows in **Figure 1**). This was not in agreement with the observation of Khan et al. (*22*), who removed catalase but not including a 62 kDa peptide from maize ICL by DEAE-cellulose column chromatography. This might be related to the different buffers and flow rates used in interlaboratories. The flow rate used in our laboratory was 0.8 mL/min, which was much faster than 10 mL/h used by Khan et al. (*22*). When the DEAE-cellulose-collected fractionations were further subjected to Sephacryl S-300 gel filtration (**Figure 3A**), most ICL activities were detected in the fractions of 60–75 tubes. Consecutively, the peanut catalase activities increased with elution volume from tube 69 to tube 79. For visualizing the protein changes during gel filtration by SDS-PAGE analysis (**Figure 3B**), changes of both enzyme activities were intimately reflected by their respective changes of band intensity. Thus, fractions of tubes 60–68 could be pooled to isolate ICL and eliminate contamination of catalase. When the pooled fraction was subjected to SDS- and native-PAGE analyses (**Figure 1**), a single ICL band was detected in both protein patterns. As shown by native-PAGE (**Figure 1B**), a single band migrated and located between bands of 440 and

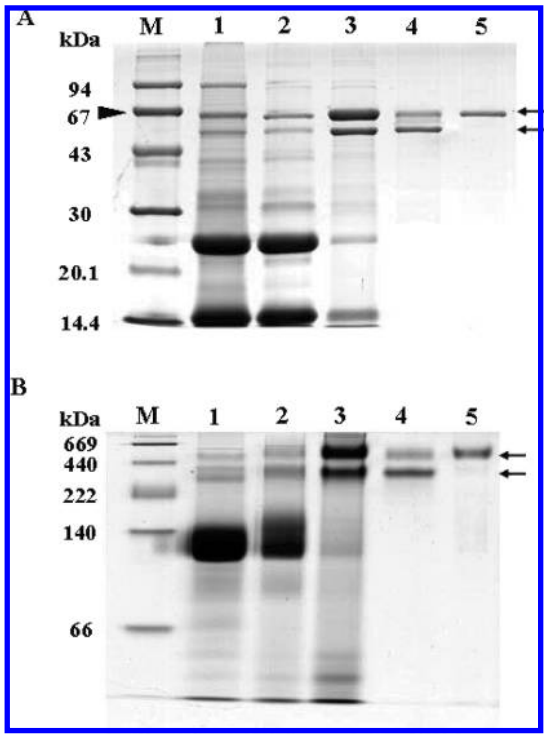

Figure 1. SDS-PAGE (**A**) and native-PAGE (**B**) analyses of the protein fractions during isocitrate lyase purification from peanut cotyledons: lane M, protein marker; lane 1, crude enzyme; lane 2, heat treatment; lane 3, 25–35% saturated ammonium sulfate precipitation; lane 4, DEAE-cellulose separation; lane 5, Sephacryl S-300 HR gel filtration.

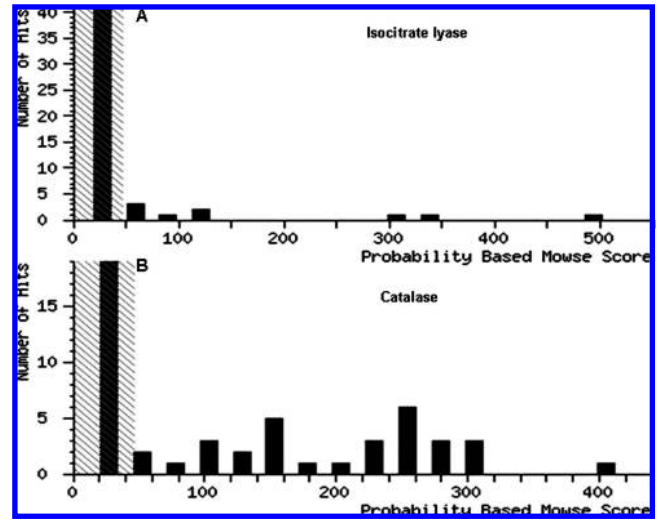

Figure 2. LC-MS/MS identification of purified peanut isocitrate lyase (ICL, **A**) and catalase (**B**) by Mascot Search based on Probability Based Morse Score.

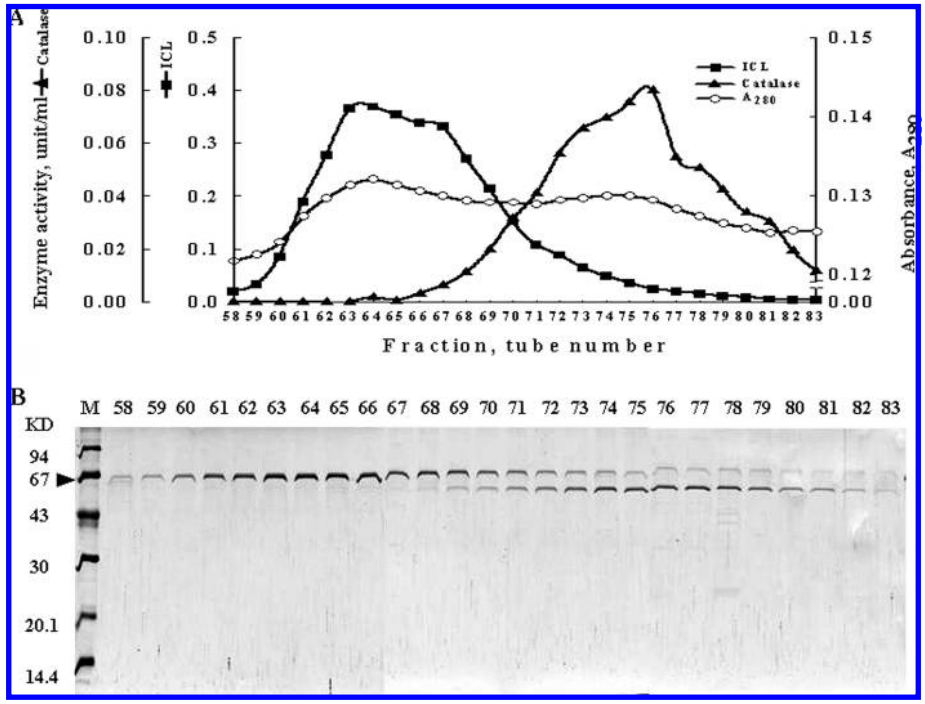

Figure 3. Changes of absorbance at 280 nm and isocitrate lyase (ICL) and catalase activity during Sephacryl S-300 HR gel filtration (**A**) and SDS-PAGE analysis of the collected fractions from 58 to 83 of the collected tubes (**B**): lane M, LWM marker; lanes 58–83, fraction tube numbers.

669 kDa. From a rough estimation of its molecular weight, it might be a complex of two tetramers of peanut ICL.

As summarized and shown in **Table 2**, a satisfactory peanut ICL purification by the involved steps was achieved. The final recovery and purity were 7.7% and 37.5-fold, respectively. The recovery was in the reported range from 1.4 to 36% as affected by the nature of the plants and methods of purification used by different laboratories (*20*). As achieved in this study, it is noteworthy that catalase could be removed from ICL by gel filtration chromatography. This was not in agreement with the observations of Yanik and Donaldson (*11*), who used a Sephacryl-300 HR column and elution with phosphate buffer (pH 8, containing 0.1 M NaCl) of the glyoxysomal matrix proteins of the castor bean seeds.

When the purified peanut ICL solutions were respectively incubated at 25, 30, 37, 45, 55, and 65 °C for activity determination (**Figure 4A**), the activities increased with an increase of temperature from 25 to 45 °C. This was in agreement with the reported characteristics of soybean and maize ICL (*21, 22*). The lower activities determined at 55 and 65 °C revealed that heat inactivation of the ICL happened at the elevated temperatures. As affected pH values ranged from 6.5 to 8.5 (**Figure 4B**), the optimal reaction pH values were 7.0 and 7.8. The optimal enzymatic pH values of soybean ICL, reacting in either phosphate or Tris-HCl buffers, are around pH 8.0 (*21*). When the purified peanut ICL solutions were heated at 25, 30, 37, 45, 55, and 65 °C for 15 min and then rapidly cooled and subjected to determination of the remained activities at 37 °C (**Figure 4C**), the ICL activities decreased sharply when the enzyme solutions were heated at temperatures above 45 °C for 15 min. As compared, even the highest ICL activity was detected at 45 °C (**Figure 4A**), but its thermal stability was poor at that temperature, in particular, when enzyme–substrate was absent. This was in agreement with the observation with soybean ICL reported by Polanowski and Obendorf (*21*).

When the peanut ICL solutions were stored at 30 °C for 8 h or at 4 °C for 15 days, >80% of the original activities were

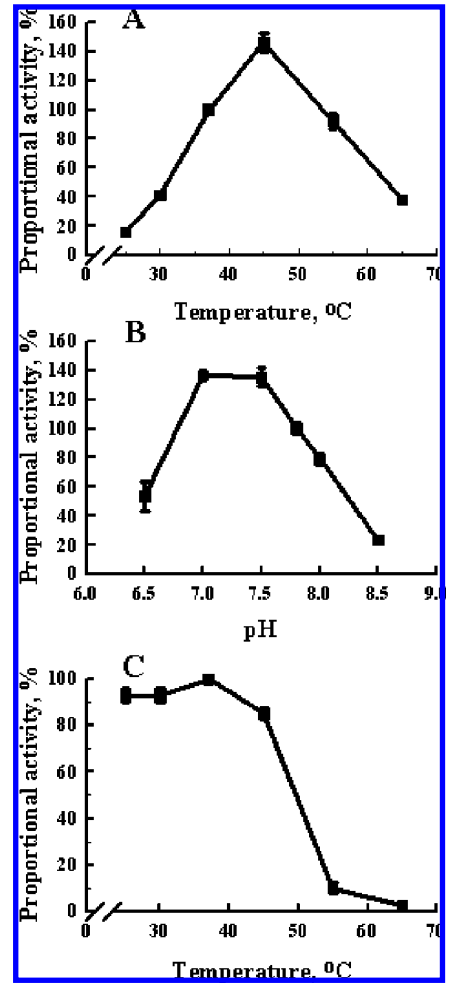

Figure 4. Enzyme activities of peanut isocitrate lyase (ICL) as affected by reaction temperature (**A**), pH value (**B**), and heat treatment at 25−65 °C for 15 min (**C**). Mean of determinations ± SD ($n = 3$).

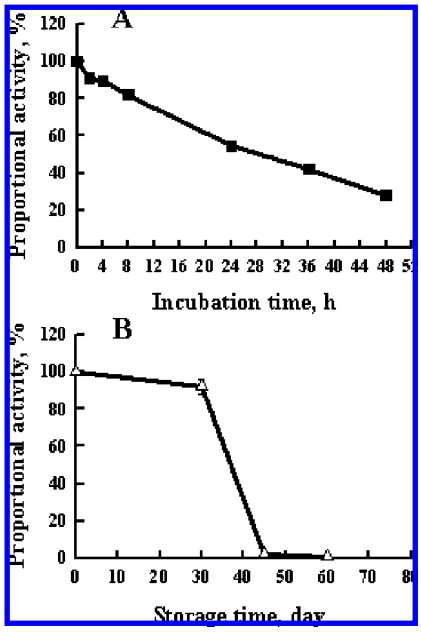

Figure 5. Enzyme activities of peanut isocitrate lyase (ICL) as affected by time of storage at 30 °C (**A**), 4 °C (**B**), and −25 °C (**C**). Mean of determinations ± SD ($n = 3$).

retained (**Figures 5**). When stored at 30 °C for 48 h, about 30% of the original activity was retained. When the enzyme was mixed with an equal volume of glycerol and stored at −25 °C for 60 days, >80% of the original activity was retained. These findings imply that peanut ICL is comparatively stable against temperature during storage.

When the peanut ICL activities were determined with supplements of various concentrations of glucose, sucrose, or ethanol in the reactants, the activities were not affected by glucose up to 4% or sucrose up to 5% (**Figures 6A**, **B**). At 7.5% of sucrose, a lower ICL activity was detected. ICL is important for gluconeogenesis of oil seeds from β-oxidation of the storage triglycerides (*1, 3*). When peanut cotyledons were cultured in 2% glucose, the developments of ICL and malate synthase were severely inhibited (*9*). However, germination of soybeans was enhanced when 147 mM sucrose was supplemented (*28*). Plant sugars are usually involved in sensing and signaling of some specific biochemical reactions (*29*). Sucrose is important in seed development and is an essential agent in driving the progress of the metabolisms (*14*). In the cell culture of cucumber, gene expression of ICL was affected by glucose, fructose, and sucrose (*16*). When the peanut ICL activities were further determined with supplements of various concentrations of ethanol in the reactants, the activities were not affected by ethanol up to 8.33% (**Figure 6C**). In comparison to control, a significantly lower activity was detected when 16.67% ethanol was supplemented. Because ethanol is commonly used for surface disinfection of plant seeds prior to germination and ICL activity is important for seed germination, caution in decreasing contact time and complete rinsing of the residual ethanol during routine practices are essential.

In conclusion, LC-MS/MS-identified peanut ICL was purified in this study by the classical steps through buffer extraction, heat treatment at 55 °C, ammonium sulfate fractionation, DEAE-cellulose chromatography, and gel filtration. During gel filtration, peanut ICL and catalase were separated. The achieved procedure in peanut ICL purification may provide a platform for further investigations of the related biochemical and physiological mechanisms of peanut kernels during germination. As affected

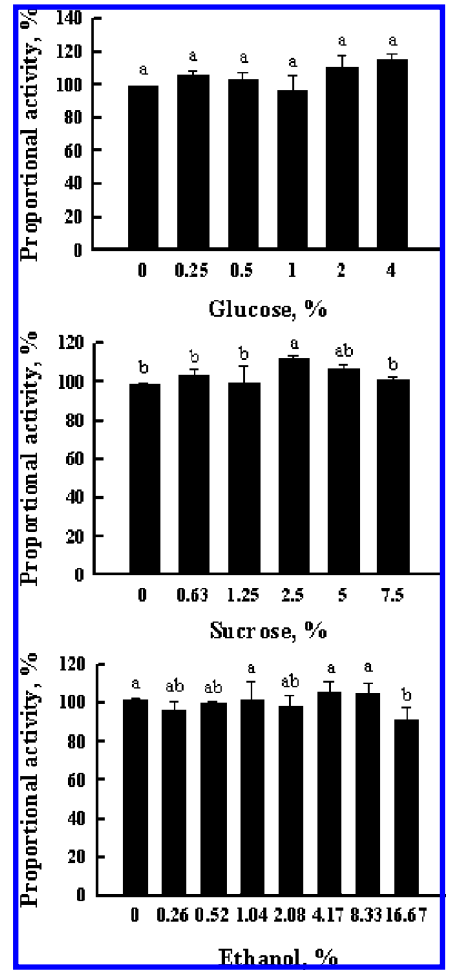

Figure 6. Enzyme activities of peanut isocitrate lyase (ICL) as affected by glucose (**A**), sucrose (**B**), and ethanol (**C**). Mean of determinations ± SD ($n = 3$); bars with different letters are significantly different ($p < 0.05$).

by supplements of glucose, sucrose, or ethanol in the reactants for activity determinations, ICL activity was not affected by glucose up to 4%, sucrose up to 5%, or ethanol up to 8.33%. Some sugars have been known to signal activation or suppress gene expression of plants (*2, 16, 30*). Ethanol treatment triggers a heat shock-like response in soybean seedlings (*17*). Under hypoxic or anoxic conditions, some plants produce energy through ethanolic fermentation (*18*), and some new physiological functions of ethanol have been raised (*31*). More sensitive up-regulation of the related gene expressions by glucose, sucrose, and ethanol deserves interest and is worthy of further investigations.

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NOTE ADDED AFTER ASAP PUBLICATION

The version published February 14, 2008, contained incorrect information in the caption to **Figures 5** and **6**. The current version of February 22, 2008, is correct.

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