Proc. 2nd Saudi Sci. Conf.,Fac. Sci., KAU. 15-17 March 2004, ***Part I,*** pp. 51-66 (2005 A.D./1425 A.H.

Expression, Purification, Properties and Quantification of Cloned Pea Choline Kinase

A. AL-MALKI*, J. L. HARWOOD** and A. MORBY**
**Department of Biochemistry, Faculty of Science, King Abdul-Aziz University, Jeddah - Saudi Arabia*
E-mail: alalmalki@kaau.edu.sa
*** School of Biosciences, Cardiff University, P.O. Box 911, Cardiff CF10 3US, Wales, UK.*

ABSTRACT. Phosphatidylcholine, the main non-chloroplastic membrane lipid is a major constituent of most plant tissues. It is synthesized in plants mainly by the cytidine diphosphate choline (CDP-choline) pathway. Choline kinase is the first enzyme in this pathway. The aim of this work was to express pea (*Pisum sativum* L. cv Feltham First) choline kinase in *E. coli* in order to facilitate its purification and characterization. It was also aimed to measure the expression levels of choline kinase gene in leaves, roots and shoots from different plant ages. Two primers designed with *Nco I* and *Bgl II* restriction sites were used to isolate a full-length cDNA coded for choline kinase from pea cDNA library. This sequence was ligated into pQE-60 vector and expressed in *E. coli* (strain DH5B4). The expressed enzyme showed a significant amount of activity. The expressed choline kinase was partially purified on nickel-nitrilotriacetic acid (Ni-NTA) column and characterized. The partially purified cloned choline kinase showed requirements for ATP and Mg^{2+} and an optimal pH in the range of 7.5 to 9.5. The K_m for choline is 6.4 x 10^{-4} M. Various potential inhibitors were tested but only O-phenanthroline had a significant effect. The expression level of the choline kinase gene was measured in leaves, roots and shoots from 4, 15 and 30 day-old peas using Real Time PCR technique. The expression was similar between tissues and did not vary much for different plant ages.

Introduction

Phosphatidylcholine is the most abundant phospholipid constituent of most plant membranes (with the notable exception of chloroplasts). It is made predominantly by the nucleotide pathway (Harwood, 1989). Choline kinase (EC 2.7.1.32) catalyses the initial step of this pathway by the ATP-dependent phosphorylation of choline to phosphorylcholine. Wittenberg and Kornberg (1953) first demonstrated choline kinase in brewer's yeast as well as in extracts of acetone powder from animal tissues.

Choline kinase has been characterized in rape seed (Ramasarma and Wetter, 1957), spinach leaves (Tanaka *et al.*, 1966) soya been (Mellor *et al.*, 1986) and castor bean

endosperm (Kinney and Moore, 1988). The enzyme has been partially purified from soya bean (Wharfe and Harwood, 1979) and from pea seedlings (Al-Malki *et al.*, 2000). It has been purified to homogeneity from animal sources (Uchida and Yamashita, 1992a; Porter and Kent, 1992).

The cDNA encoding choline kinase has been cloned from rat liver and kidney (Uchida, 1994; Aoyama *et al.*, 1998), yeast (Hosaka *et al.*, 1989), soya bean (Monks *et al.*, 1996) and pea (Al-Malki *et al.*, 2000). In soya bean, three cDNAs were found, two of whieh had full length reading frames coding for proteins of 359 and 362 amino acid residues (Monks *et al.*, 1996). In peas, a cDNA eoding for a protein of 343 amino aeid residues was found (Al-Malki *et al.*, 2000). The soya bean choline kinase has been expressed in yeast and *E. coli* and exhibited a signifieant amount of aetivity (Monks *et al.*, 1996).

Previously we expressed the eDNA coding for choline kinase from pea in TOPO vector to prove the identity of the gene (Al-Malki *et al.*, 2000). The present paper deseribes the expression of this gene into *E. coli* using QIAexpress system in order to facilitate the purification and eharacterization of the expressed protein. The quantification of the pea choline kinase gene in roots, shoots and leaves is also described.

Materials and Methods

Cloning of the full length pea choline kinase into pQE-60 vector

Primer design and polymerase chain reaction (PCR) conditions to amplify the choline kinase open reading frame

Two primers (PQEF and PQER) were designed from the sequence of pea choline kinase to amplify the entire coding region. The sequences of the primers were (forward: 5'-GGC CAT GGC TTT AAA AAC CTT TGA ATT GTT GA-3' and reversc: 5'-GGA AGA TCT GGC AAC AAA ATA ACT TTC TTT AAC CAG-3'). The two primers contained restriction sites *Nco I* for the 5'-end and *Bgl II* for the 3'-end. These primers were used to amplify the full-length sequence of pea choline kinase from pea cDNA library.

PCR was performed in a 20 µl reaction volumes. One microliter aliquots of DNA lysate werc used as a template. PCR reactions were hot started by adding all componcnts except *Taq* DNA polymerase, heating to 95°C for 10 min and then holding at 80°C for 30 min. *Taq* DNA polymerase (1.5 units) was added and amplification was performed over 32 cycles. The cycles were usually 95°C for 1 min, 54°C for 1 min and 72°C for 2 min. Reactions were performed in a programmed thermocycler (Primus 25 HE, MWG-BIOTECH). The PCR products were then separated by clectrophoresis on a 2% agarose gels.

Cloning of the full length choline kinase cDNA into pGEM-T vector

The PCR product obtained was ligated into pGEM-T vector. The ligation mix was used to transform *E. coli* JM109 eells. Positive colonies were selected and checked for the correct orientation, and then were sequenced.

Ligation of the pea cDNA clone into pQE-60 vector

Both the PCR product and pQE-60 vector were digested with the restriction enzymes *Nco I* and *Bgl II*. The linearized vector and choline kinase were purified and ligated. The ligated DNAs were transformed into *E. coli* (DHB4).

Expression of choline kinase into pQE-60 vector

E. coli cells (strain DHB4) transformed with pea choline kinase were cultured in 10ml of Luria broth medium (containing ampicillin) to an absorbance of 0.5 – 0.7 at 595nm. The expression was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 100μM. After 18 hours, the cells were harvested by centrifugation and resuspended in 10ml of phosphate buffer (pH 8.0). The cells were then lysed by using a French press and debris was pelleted by centrifugation at 100,000 *x g* for 60 minutes. The soluble fraction was used for choline kinase purification.

Partial purification of cloned choline kinase

A nickel-nitrilotriacetic acid (Ni-NTA) column was used to purify his-tagged cloned choline kinase. The column was equilibrated with 50 mM phosphate buffer (pH 8.0) containing 0.1 M NaCl. Total proteins of 1.6 mg were loaded onto the column. The column then washed with 3 volumes of 50 mM phosphate buffer (pH 8.0) containing 0.1 M NaCl. Choline kinase was then eluted with a continuous gradient of 0.1 – 0.5 NaCl in the washing buffer. Fractions of 1.5 ml were collected and assayed for choline kinase activity.

Choline kinase assay

Choline kinase activity was assayed according to the method of Uchida and Yamashita (1992b) with slight modifications. The reaction mixture contained 50 mM bicine buffer (pH 8.7), 10 mM ATP, 12 mM $MgCl_2$, 15 mM KCl, 3 mM choline chloride containing 0.04 μCi [methyl-^{14}C]choline chloride and the fraction to be assayed in a total volume of 100 μl. The reaction was started by the addition of the enzyme and normally carried out at 30°C for 45 min. The reaction was terminated by the addition of 250μl of tetraphenylboron solution (30 mg/ml in butyronitrile), followed by vigorous mixing on a vortex. The tube was centrifuged at 1,000 *x g* for 2 min. The upper phase was aspirated off and the lower phase was washed 3 times with the tetraphenylboron solution to remove unreacted [methyl-^{14}C]choline, transferred to a counting vial and counted in the presence of Opti-fluor scintillant. Radioactivity was determined directly by suspending the sample in 10ml Opti-fluor scintillant (Canberra Packard) in plastic scintillation vials. Samples were usually counted for one minute. Radioactivity was counted using an LKB Wallac 1209 Rackbeta liquid scintillation counter (Wallac Oy, Turku, Finland).

Protein determination

Protein was determined according to the method of Bradford (Bradford, 1976). Samples contained between 10μg and 140μg of proteins in a volume of less than 100μl were measured according to the standard Bradford assay, while samples containing 1μg to 10μg proteins were measured according to the micro Bradford assay.

Real Time PCR

The expression level of the choline kinase gene was measured using the LightCycler (Idaho Technology-LC24). The reaction mixture used for Real Time PCR contained 2mM/µl deoxynucleotide triphosphates, 3 mM/µl $MgCl_2$, 10 pmol/µl for each of the forward and reverse primers, (Syber Green) and (1U/µl) *Taq* DNA polymerase in a total volume of 200µl. one µl of DNA was added to 9µl of the PCR mixture and mixed. Five µl of the reaction mixture was added to the LightCycler capillary (Biogen Ltd, Cambridge, UK) and centrifuged at low speed for 5 sec.

The LightCycler capillaries were then sealed and loaded onto the LightCycler and subjected to a PCR programme. The PCR programme was carried out over 32 cycles and usually 95°C for 1 second (denaturation), 55°C for 3 seconds (annealing), 72°C for 10 seconds (elongation) followed by 94°C for 1 sec.

Results and discussion

Cloning and partial purification of pea choline kinase

Two primers (PQEF and PQER) were designed from pea choline kinase sequence to amplify the cDNA coding for choline kinase from λgt10 library. The primers sequence were (PQEF: 5'-GGC CAT GGC TTT AAA AAC CTT TGA ATT GTT GA-3' and PQER: 5'-GGA AGA TCT GGC AAC AAA ATA ACT TTC TTT AAC CAG). The two primers contained restriction sites *Nco I* for the 5'-end and *Bgl II* for the 3'-end. The expected size of the PCR product, based on the full-length sequence was 1032 bp (Al-Malki *et al.*, 2000). Analysis of the amplified cDNA on 2% agarose gel electrophoresis (Figure 1) showed a single band of the expected size.

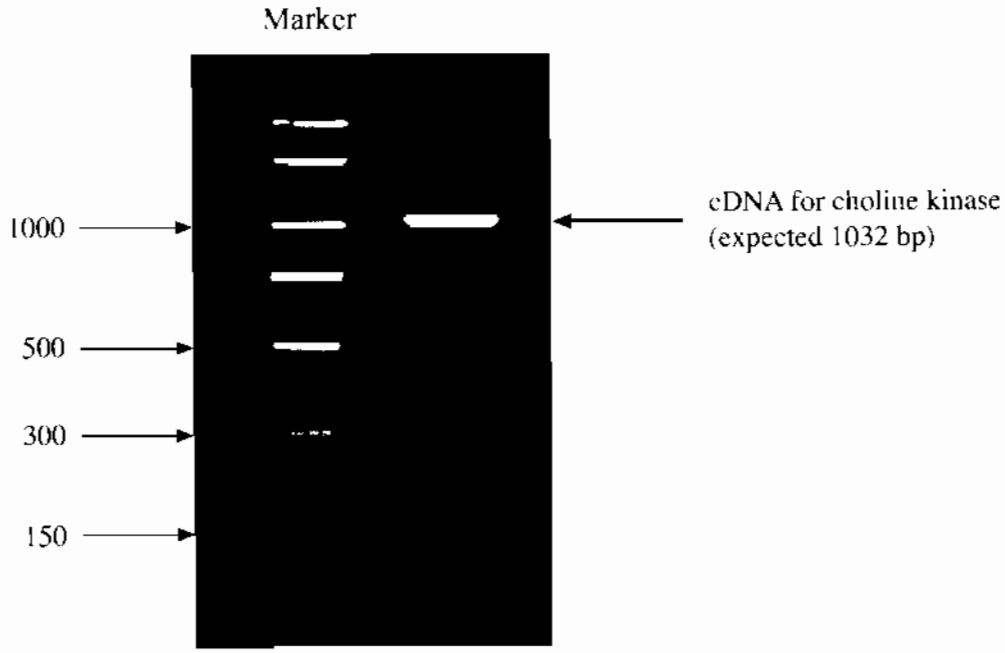


Fig. (1). Separation of PCR product using specific primers designed from pea choline kinase sequence with restriction sites.

The PCR product obtained was excised from the gel and purified. The purified DNA was ligated into pGEM-T vector and used to transform *E.coli* JM109 cells. Colonies were PCR-screened and positive colonies were selected and checked for the correct orientation. The sequence was checked for any possible mutations introduced by the PCR and no mutations were found anywhere in the entire sequence. A double digest was performed on the colony selected using *Nco I* and *Bgl II* restriction enzymes. Both enzymes digested suecessfully to give a product of about 1032 bp (Figure 2).

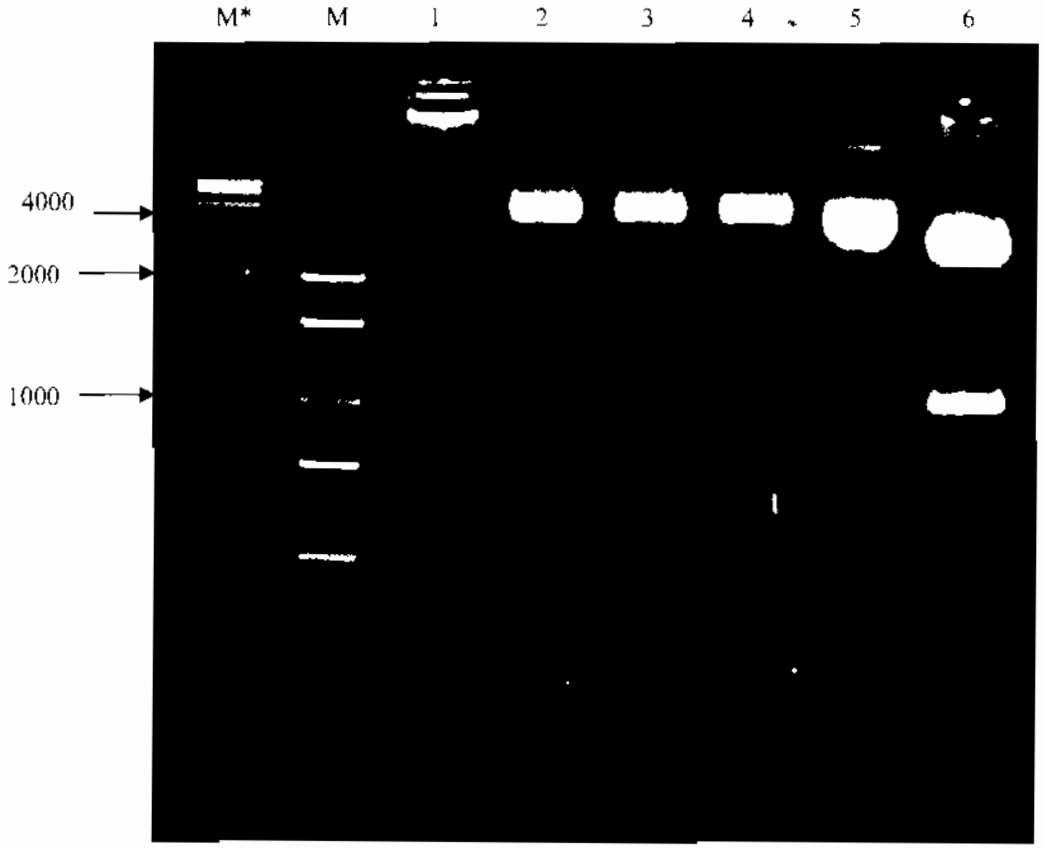


Fig. (2). Restriction digestion of pQE-60 expression veetor and pea choline kinase cDNA cloned into pGEM-T vector with Nco I and Bgl II as visualised by 1.2% agarose gel electrophoresis

1 = undigested pQE-60 vector
2,3 & 4 Digested pQE-60 vector
5 pea choline kinase cDNA cloned into pGEM-T vector and not digested
6 pea choline kinase cloned into pGEM-T vector and digested
M* = 10,000 bp ladder and M 50-2000 bp DNA ladder.

pQE-60 vector and cloned pea choline kinase cDNA were digested with the restriction enzymes *Nco I* and *Bgl II*. The restriction enzymes cut both the cloned choline kinase cDNA and pQE-60 vector successfully (Figure 2). The pea choline kinase cDNA and the pQE-60 vector were ligated and used to transform *E. coli* (DHB4) cells. The transfonuants were PCR-screened using primers designed from the pQE-60 vector sequence. A band of about 1.2 kb, which was represent the entire coding region of pea choline kinase cDNA (1032 bp) plus part of the veetor sequence, was obtained.

A positive clone was used to inoculate 10 ml of LB medium containing 50 µg/ml ampicillin. The culture was incubated overnight at 37°C with shaking. Five ml of the overnight culture was used to inoculate 10 ml of LB medium containing 50 µg/ml ampicillin and incubated at 37°C with shaking until an absorbance of about 0.6 (OD_{595})

was reaehed. Expression was indueed by the addition of 100 μM IPTG (final concentration) and the culture grown at 25°C for 18 hours. The cells were harvested by centrifugation and then resuspended in 10 ml of phosphate buffer (pH 8.0). The cells were then lysed by using a French press and debris was pelleted by centrifugation at 100,000 *x g* for 60 minutes. The time- course of expression of pea choline kinase activity in (DHB4) cells is shown in Figure 3.

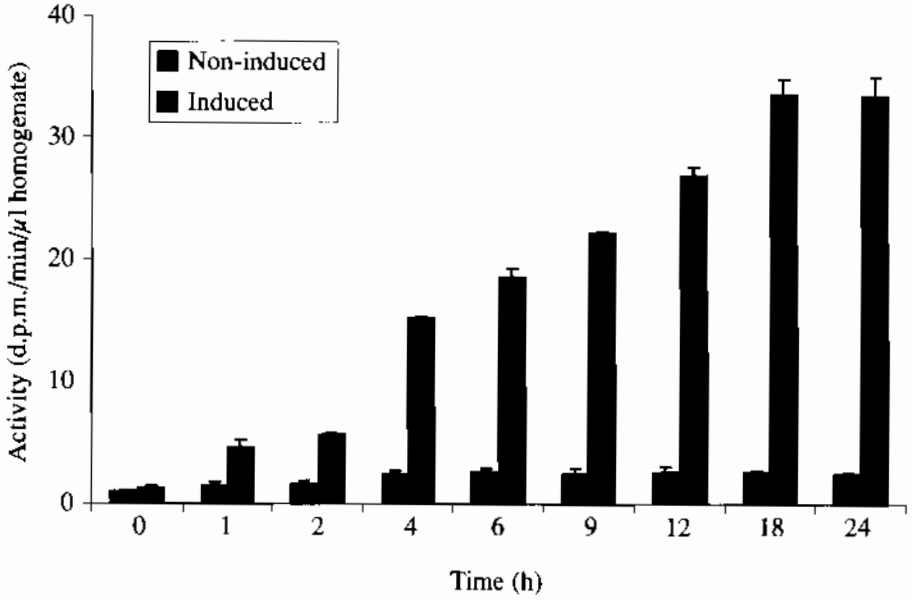


Fig. (3). Time-course of expression of pea choline kinase activity in DHB4 cells. Induced samples were suspended in 100 μl of 50 mM bicine buffer (pH 8.7). aliquots (5 μl) were assayed for choline kinase activity.

Results are mean ± SD, where n= 3

Cloned pea choline kinase was partially purified using immobilized-metal affinity chromatography (IMAC). Porath *et al* (1975) first used IMAC to purify proteins using the chelating ligand iminodiacetic acid (IDA). IDA was charged with metal ions such as Zn^{2+}, Cu^{2+}, or Ni^{2+} and then used to purify different proteins and peptides (Sulkowski, 1985). This method was improved by using a tetradentate chelating absorbent called nitrilotriacetic acid (NTA). NTA ligand binds nickel ion to form Ni-NTA.

To purify cloned pea cholinc kinase, 100 □l of the soluble crude extract were loaded onto 1 ml Ni-NTA column, which had been previously equilibrated with loading buffer (50 mM phosphate buffer (pH 8.0) containing 0.3 M NaCl and 10 mM imidazole). Thc proteins were then eluted with a continuous gradient of 10-25 mM imidazole in 50 mM phosphate buffer (pH 8.0) containing 0.3 M NaCl. Fractions of 0.5 ml were colleeted and assayed for eholine kinase aetivity. The results showed that choline kinase was eluted in imidazole gradient but was associated with many other proteins.

Characterization of partially purified cloned choline kinase

The partially purified cloned choline kinase was used to study the characteristics of the enzyme.

Figure 4 and 5 showed that the maximal aetivity of the pea eloned choline kinase occurred when the concentration of ATP and $MgCl_2$ were 10 mM. The equimolar ratio was also reported for rat liver (Weinhold and Rethy, 1974), rapeseed (Tanaka *et al*. 1966), rat

brain (Uchida and Yamashita, 1990), soya bean (Wharfe and Harwood, 1979) and pea seedling choline kinase (Al-Malki *et al.* 2000). For both ATP and $MgCl_2$ concentrations higher than 10 mM resulted in less activity. These effects may be due to a stimulation of a competing enzyme or, possibly, to a direct action (e.g. salt-induced conformation changes) on choline kinase.

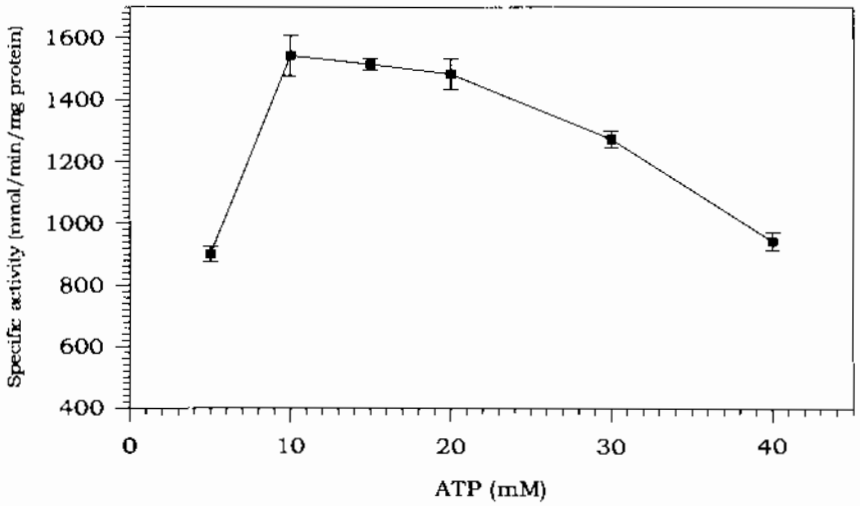


Fig. (4). Effect of ATP concentration on cloned choline kinase activity. Assay was performed at 30°C for 30 minutes. $MgCl_2$ (10 mM) was used in the assay mixture.

Results are mean ± SD, where n=3 replicates.

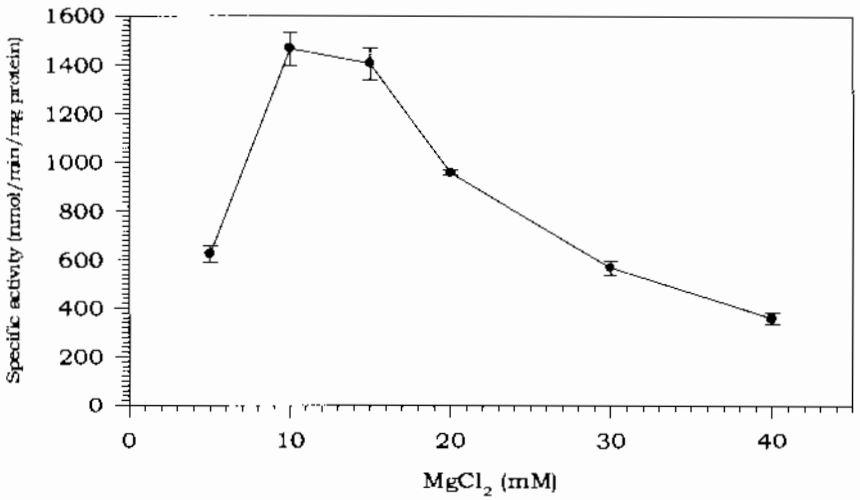


Fig. (5). Effect of $MgCl_2$ concentration on the activity of cloned choline kinase. The assay was carried out at 30°C for 30 minutes. ATP (10 mM) was used.

Results are mean ± SD, where n=3 replicates.

Figure 6 shows that the activity of cloned choline kinase using different buffer systems gave a broad pH optimum in the range 7.5 to 9.5. The result obtained agreed with data reported by Tanaka *et al.* (1966) for spinach leaves where they found the optimum pH broad in the range of 7.5 to 10.5 (according to the type of the buffer used). Optimal pH values of around pH 8.7 was found in other studies (Ramasarma and Wetter, 1957; Wharfe and Harwood, 1979).

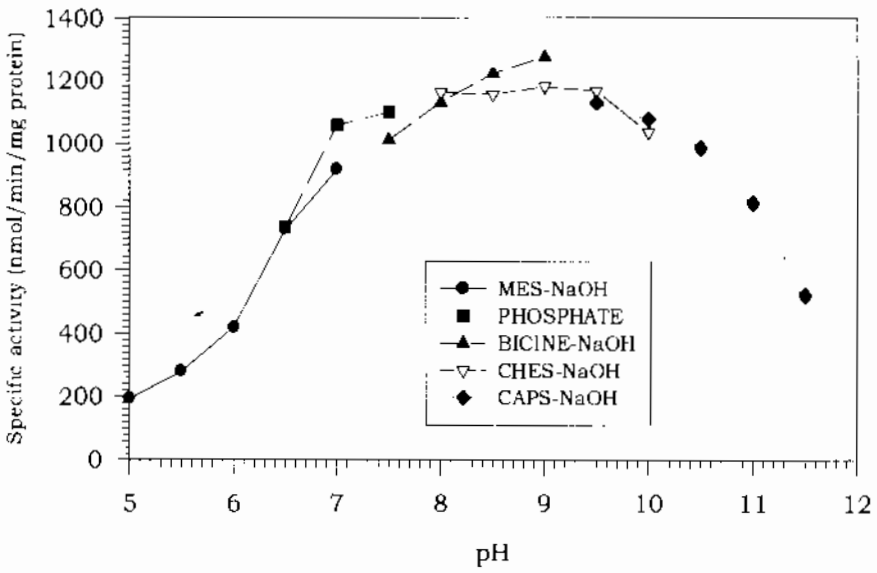


Fig. (6). Effect of pH on the activity of cloned choline kinase. The activity was measured using different buffers at final concentrations of 20 mM. The reaction was carried out at 30°C for 30 minutes. ATP and $MgCl_2$ were used at final concentrations of 10 mM.

The results are mean ± SD, where n 3 replicates.

Different concentrations of choline chloride were used to measure choline kinase activity. Figure 7 shows that the maximal activity of cloned choline kinase was achieved at about 3 mM choline chloride. This result was also obtained with pea seedling choline kinase (Al-Malki *et al.* 2000). The data yielded a K_m of 6.4 x 10^{-4} M. This also agreed with previous result from non-plant tissues (Uchida and Yamashita, 1990). 5 mM choline was also used to measure activity in soya bean and yeast (Wharfe and Harwood, 1979; Kim *et al.* 1998).

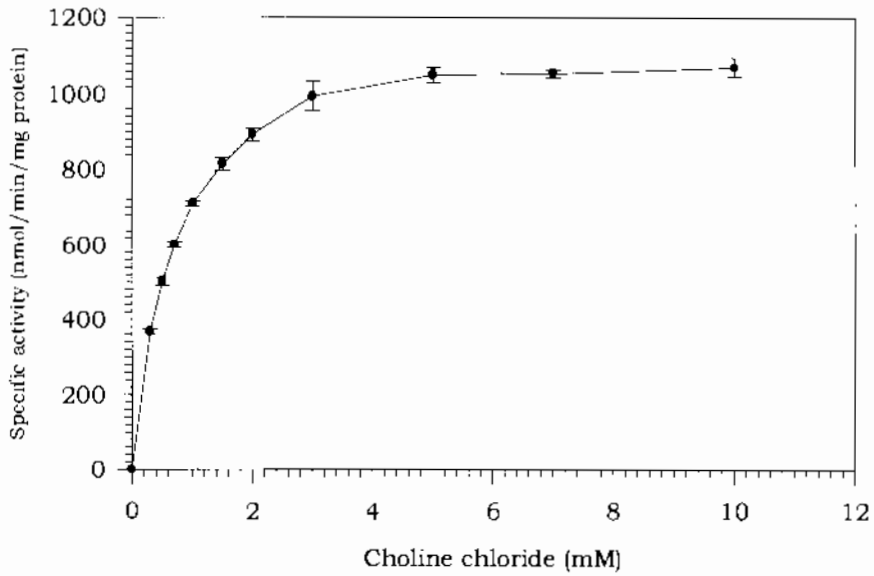


Fig. (7). Effect of different concentrations of choline chloride on the activity of cloned choline kinase. ATP and $MgCl_2$ were used at final concentrations of 10 mM. Bicine buffer used at a pH of 9.0.

Results are mean ± SD, where n=3 replicates.

Some possible enzyme inhibitors were tested for their effect on the activity of cloned choline kinase. O-phenanthroline and indole-3-acetic acid (IAA) were added at a final concentration of 1 mM. EGTA was added at a final concentration of 5 mM. EGTA had no effect on the enzyme activity indicating that Ca^{2-} was not required. Although IAA appeared to inhibit the enzyme activity poorly, there was no significant difference between the control and IAA. O-phenanthroline inhibited cloned choline kinase activity significantly (P = 0.027) by about 24% (Figure 8). This suggested that iron might be involved in the enzyme's activity. The lack of effect of an –SH inhibitor such as IAA agrees with other work with choline kinase (Ramasarma and Wetter, 1957; Tanaka *et al.*, 1966).

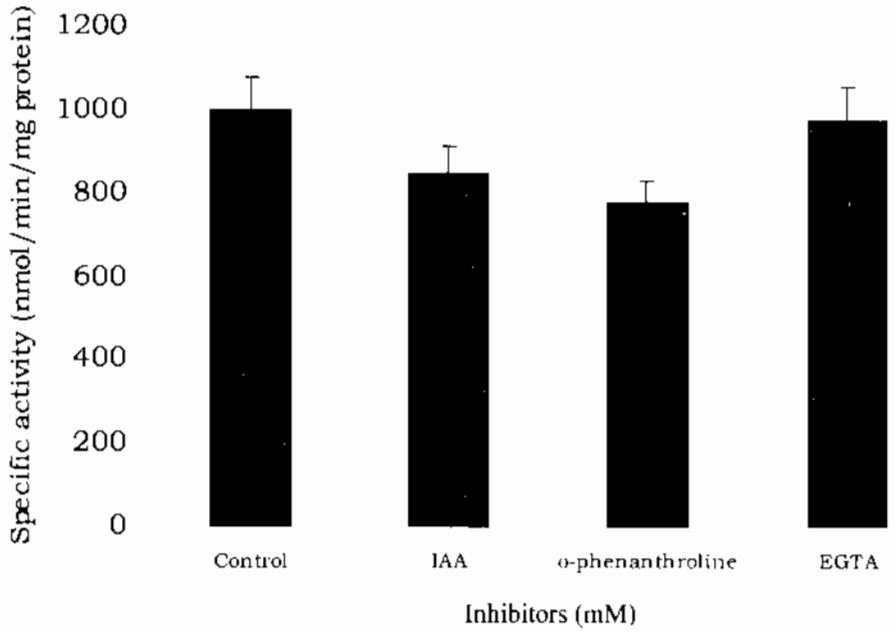


Fig. (8). Effect of some potential enzyme inhibitors on the activity of the cloned enzyme. The inhibitors were pre-incubated with the enzyme preparation for 10 minutes at 30°C. The reaction was then started by the addition of ^{14}C-choline and carried out for 30 minutes. ATP and $MgCl_2$ were used at final concentrations of 10 mM. Bicine buffer (pH 9.0) and 3 mM of choline chloride were also used.

IAA was used at a concentration of 1mM
O-phenanthroline was used at a concentration of 1mM
EGTA was used at a concentration of 5mM

Results are mean ± SD, where n=3 replicates.

Hemicholinium-15 is known to inhibit the activity of choline kinase. Figure 9 showed that hemicholinium-15 was a strong inhibitor for the cloned choline. Addition of 0.001 mM of the inhibitor resulted in a significant inhibition of the enzyme activity (P = 0.016). Moreover, 10 mM of the inhibitor resulted in greater than 95% inhibition of cloned choline kinase activity (P = 0.0031). These data were in agreement with data obtained for the effect of the inhibitor on the incorporation of radiolabelled choline into phosphatidylcholine (unpublished data). These data also agreed with previous results obtained from soya bean (Dykes *et al.* 1976) and suggested that hemicholinium-15 was a potent inhibitor of pea choline kinase activity.

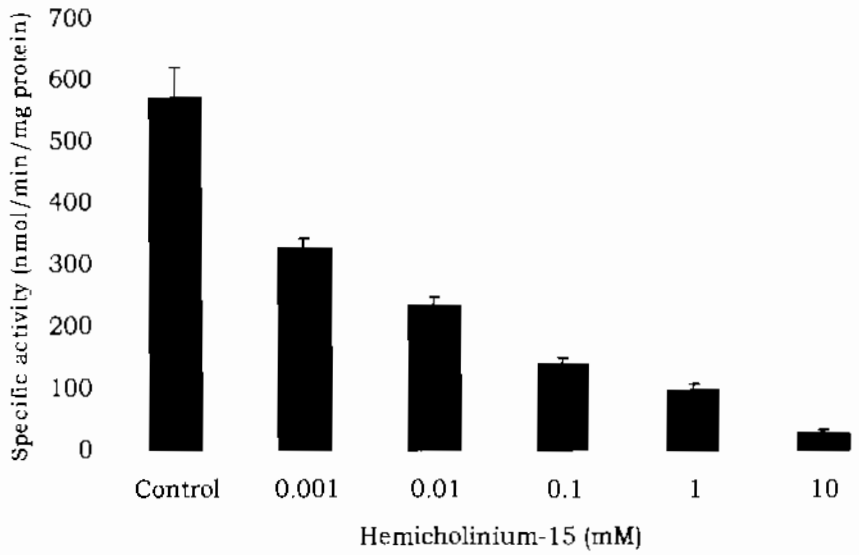


Fig. (9). Effect of hemicholinium-15 on the activity of cloned choline kinase. Hemicholinium-15 was pre-incubated with the enzyme preparation for 10 minutes at 30°C. The reaction was then started by the addition of ^{14}C-choline and carried out for 30 minutes at 30°C. ATP and $MgCl_2$ concentrations were 10 mM. Bicine buffer (pH 9.0) and of choline chloride (3 mM) were used.

Results are mean ± SD, where n=3 replicates.

Quantification of pea choline kinase cDNA

RNA was isolated from three tissues (roots, shoots and leaves) and the expression levels of the pea choline kinase gene were quantified by Real Time PCR using a LightCycler™ (Idaho Technology, Idaho Falls, USA). This method allows fluorescent monitoring of rapid cycle DNA amplification (Wittwer *et al.*, 1997a), template quantification (Wittwer *et al.*, 1997b) and product examination by melting curve analysis (Ririe *et al.*, 1997).

The LightCycler is a sensitive technique with a large number of applications in molecular biology. With regard to quantification, fluorescent dyes can be used to quantify nucleic acids and double-strand denaturation in order to allow for detection and monitoring of dsDNA amplification (Wittwer *et al.*, 1997b). Ethidium bromide and Syber Green are the most useful dsDNA-specific dyes that can be used to detect DNA. By using these dyes with fine template control, PCR can be monitored cycle by cycle and product purity can be estimated by melting curve analysis without the need for end-point analysis by agarose gel electrophoresis (Wittwer *et al.*, 1997a).

Preparation of cDNA for the LightCycler

Total RNA was prepared from pea roots, shoots and leaves after 4, 15 and 30 days growth period using RNeasy Plant Mini Kit (Qiagen). Equivalent amounts of total RNA (240 μg) were used to prepare mRNA using an Oligotex mRNA purification Kit (Qiagen). mRNA was then used to prepare cDNA in a reverse transcription process.

Construction of the Standard Curve for 18S rRNA

A standard curve for the 18S rRNA was constructed utilizing the primers (forward: 5'-ATG GTG GTG ACG GGT GAC-3' and reverse: 5'-GCC CAA GGT CCA ACT ACG-

3'). Plasmid DNA containing the transcript was prepared and adjusted to 1ng/µl and then different concentrations were prepared and amplified using Real Time PCR to construct the standard curve for 18S rRNA. Figure 10 shows the quantitative amplification of a standard dilution series from the 18S rRNA (Panel A) and the standard curve for the 18S rRNA construct (Panel B) which was subsequently used to quantify expression of the gene.

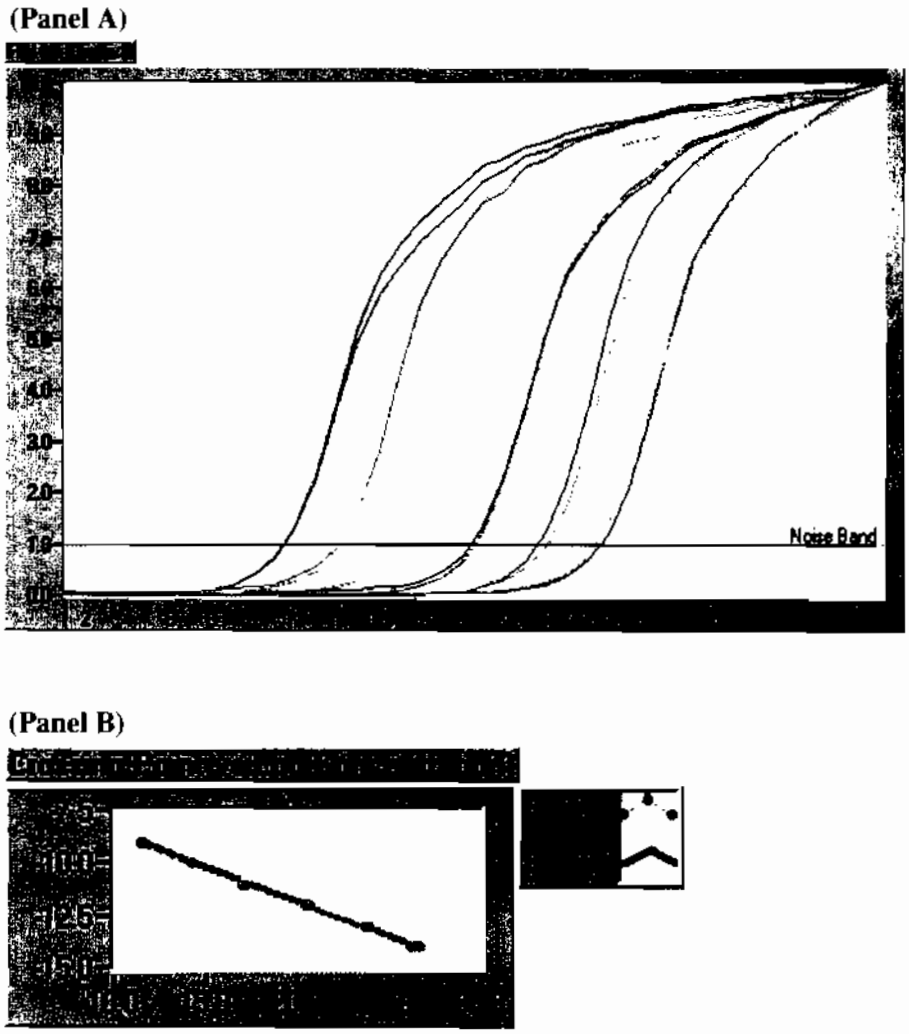


Fig. (10). (Panel A) Quantitative amplification of a standard dilution series from the 18S rRNA transcript. The template concentrations of each standard were 1ng/µl, 100pg/µl, 10pg/µl, 1pg/µl, 100fg/µl and 10fg/µl. (Panel B) standard curve for the 18S rRNA construct used to quantify the expression of 18S gene with the LightCycler.

Construction of the Standard Curve for choline kinase

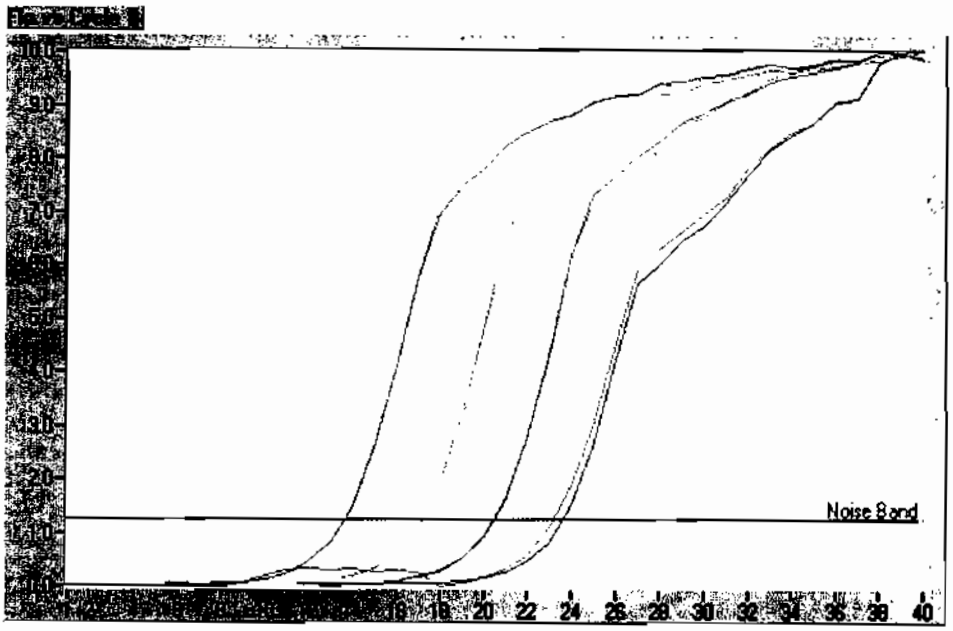
Two primers (forward: 5'-GAG ATA AAT TCG CTG AGG GAG-3' and reverse: 5'-TGC AGC CAT TTC ACA GAA GTG-3') were used to construct the standard curve for choline kinase. Plasmid DNA which contained the transcript was prepared and adjusted to 1ng/µl. Serial dilutions were then carried out to prepare a standard curve for choline kinase. Figure 11 shows the quantitative amplification of a standard curve for choline kinase (Panel A) and the standard curve of choline kinase (Panel B). The standard curve was used subsequently to quantify expression of the choline kinase gene.

Quantification of the pea 18S rRNA expression by Real Time PCR

In order to standarise the expression level of the gene of interest (choline kinase), the expression level of the 18S rRNA (the control) was measured for each cDNA sample. The cDNA samples investigated were from pea (*Pisum sativum* L. cv Feltham first) roots, shoots and leaves from pea which had been germinated for ages 4, 15 and 30 days.

The LightCycler products were found to be specific to 18S rRNA. The values for 18S rRNA from roots, shoots and leaves at pea ages of 4, 15 and 30 days were obtained from the standard curve for 18S rRNA. These values were used to standarise gene expression quantified to copy number per µg of the 18S rRNA.

(Panel A)



(Panel B)

Fig. (11). (Panel A) Quantitative amplification of a standard dilution series from choline kinase transcript. The template concentrations in each standard were 100pg/µl, 10pg/µl, 1pg/µl and 100fg/µl. (Panel B) Staudard curve for choline kinase construct nsed to quantify the expression of choline kinase gene with the LightCycler.

Quantification of the pea choline kinase expression by Real Time PCR

Gene expression of choline kinase in pea roots, shoots and leaves at ages 4, 15 and 30 days were quantified. Melting curves (Figure 12a, b, c) showed only specific products were obtained with uo primer-dimers. From the values obtained from the standard curve, the copy numbers for choline kinase were calculated and standarised to copy numbers / μg 18S rRNA. The expression level of pea choline kinase was rather similar between the different tissues. Furthermore, choline kinase gene is thought to be a housekeeping gene because of its wide distribution between tissues (Uchida, 1994). Thus, one would not expect the gene to show inducible characteristics but to be well expressed throughout the plant's active growing period.

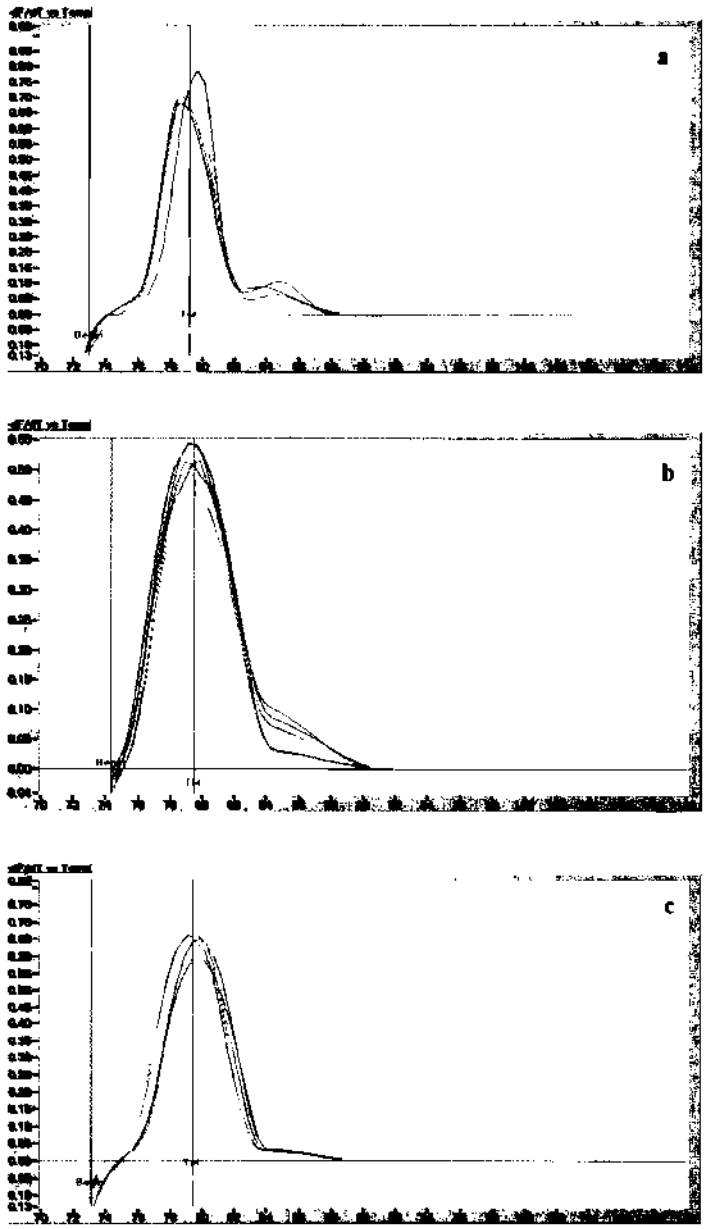


Fig. (12). Melting curves for choline kinase cDNA amplification by the LightCycler on pea roots(a), shoots(b) and leaves(c) respectively after 4 days, 15 days and 30 days of germination.

References

Al-Malki A., Morby A. and **Harwood J.L.** (2000) *Pea choline kinase: purification, properties and isolation of a cDNA.* In Recent Advances in the Biochemistry of Plant Lipids (Edited by J. L. Harwood and P. J. Quinn) Portland Press (London) pp 721-723.

Aoyama, C., Nakashima, K., Matsui, M. and **Ishidate, K.** (1998) Complementary DNA sequence for a 42 kDa rat kidney choline/ethanolamine kinase. *Biochim. Biphys. Acta.* **1390:** 1-7.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.*, **72:** 248-254.

Dykes, C.W., Kay, J. and **Harwood, J.L.** (1976) Incorporation of choline and ethanolamine into phospholipids in germinating soya bean. *Biochem. J.* **158:** 575-581.

Harwood, J.L. (1989) Lipid metabolism in plants. *Crit. Rev. Plant Sci.* **8:** 1-43.

Hosaka, K., Kodaki, T. and Yamashita, S. (1989) Cloning and characterisation of the yeast *CKI* gene encoding choline kinase and its expression in *Escherichia coli. J. Biol. Chem.*, **264:** 2053-2059.

Kim, K.H., Voelker, D.R., Flocco, M.T. and **Carman, G.M.** (1998) Expression, purification and characterisation of choline kinase, product of the *CKI* gene from *Saccharomyces cerevisiae. J. Biol. Chem.*, **273:** 6844-6852.

Kinney, A.J. and **Moore, Jr. T.S.** (1988) Phosphatidylcholine synthesis in castor bean endosperm: the localisation and control of CTP: choline-phosphate cytidylyltransferase activity. *Arch. Biochem. Biophys.*, **259:** 15-21.

Mellor, R.B., Christensen, T.M.I.E. and **Werner, D.** (1986) Choline kinase II is present only in nodules that synthesise stable peribacteroid membranes. *Proc. Natl. Acad. Sci. USA,* **83:** 659-663.

Monks, D.E., Goode, J.H. and **Dewey, R.E.** (1996) Characterisation of soybean choline kinase cDNA and their expression in yeast and *Escherichia coli. Plant Physiol.*, **110:** 1197-1205.

Porath, J., Carlsson, J, Olsson, I. and **Belfrage, G.** (1975) Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature,* **258:** 598-599.

Porter, T.J. and **Kent, K.** (1992) Choline/ethanolamine kinase from rat liver. *Methods in Enzymol.*, **209:** 134-146.

Ramasarma, T. and **Wetter, L.R.** (1957) Choline kinase of rapeseed (*Brassica campestris* L.). *Can. J. Biochem. Physiol.* **35:** 853-863.

Ririe, K.M., Rasmussen, R.P. and **Wittwer, C.T.** (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem.* **245:** 154-160.

Sulkowski, E. (1985) Purification of proteins by IMAC. *Trends Biotechnol.* **3:** 1-7.

Tanaka, K., Tolbert, N.E. and **Gohlke, A.F.** (1966) Choline kinase and phosphorylcholine phosphatase in plants. *Plant Physiol.*, **41:** 307-312.

Uchida, T. (1994) Regulation of choline kinase R: analysis of alternatively spliced choline kinase and the promoter region. *J. Biochem.*, **116:** 508-518.

Uchida, T. and **Yamashita, S.** (1990) Purification and properties of choline kinase from rat brain. *Biochim. Biophys. Acta.* **1043:** 281-288.

Uchida, T. and **Yamashita S.** (1992a) Molecular cloning, characterisation and expression in *Eacherichia coli* of a cDNA encoding mammalian choline kinase. *J. Biol. Chem.*, **267:** 10156-10162.

Uchida, T. and **Yamashita S.** (1992b) Choline/ethanolamine kinase from rat brain. *Methods in Enzymol.*, **209:** 147-153.

Weinhold, P.A. and **Rethy, V.B.** (1974) The separation, purification and characterisation of ethanolamine kinase and choline kinase from rat liver. *Biochemistry,* **13:** 5135-5141.

Wharfe, J. and **Harwood, J.L.** (1979) *Purification of choline kinase from soya bean.* In Advances in Biochemistry and Physiology of Plant Lipids (Appelqvist, L. A., and Liljenberg, C., eds.), pp. 433-447, Elsevier / Scientific Publications, Amsterdam.

Wittenberg, J. and **Kornberg, A.** (1953) Choline phosphokinase. *J. Biol. Chem.*, **202:** 431-444.

Wittwer, C.T., Herrmann, M.G., Moss, A.A. and **Rasmussen, R.P.** (1997a) Continuous fluorescence monitoring of rapid cycle DNA amplification. *BioTechniques,* **23:** 130-138.

Wittwer, C.T., Ririe, K.M., Andrew, R.V., David, D.A., Gundry, R.A. and **Balis, U.J.** (1997b) The LightCycler™: a microvolume multisample fluorimeter with rapid temperature control. *BioTechniques,* **22:** 176-181.

تنقية ودراسة خصائص وكمية كولين كاينـاز البازلاء المستنسخ

عبد الرحمن المالكي*، جون هاروود و أندي موربي****

*قسم الكيمياء الحيوية ، كلية العلوم ، جامعة الملك عبد العزيز

جـــــــده – المملكة العربية السعودية

البريد الإلكتروني : alalmalki@kaau.edu.sd

**مدرسة العلوم البيولوجية ، جامعة كاردف ، ويلز – المملكة المتحدة*

المستخلص. يعتبر الفوسفاتيدل كولين المكون الرئيسي لدهون الأغشية غير الكلوروبلاستيدية، وهو المكون الرئيسي لأغلب الأنسجة النباتية. يخلق الفوسفاتيدل كولين في النباتات بمسار السايتيدين ثنائي الفوسفات – كولين. ويعتبر الكولين كايناز الإنزيم الأول في هذا المسار. الهدف من هذه الدراسة هو استنساخ كولين كايناز في بكتيريا الاشريشيا كولاي وذلك لتسهيل تنقيته ودراسة خواصه. كما تهدف الدراسة أيضا إلى قياس مستويات التعبير عن جين كولين كايناز في الأوراق و الجذور والطلائع في مراحل عمرية مختلفة للنبات. صممت في البداية بادئتين تحويان على موقعي قيد (حصر) هما إن. سي. أو ١ (*Nco I*) و ب. ج. أل ٢ (*Bgl II*) واستخدمتا لعزل الدنا المتمم (cDNA) كامل الطول والمشفر للكولين كايناز من مكتبة الدنا المتمم للبازلاء. تم ربط هذا التسلسل بناقل بي. كيو. أي ٦٠ (pQE-60) وتم التعبير عنه في البكتيريا (سلالة دي. اتش. بي ٤ ، DHB4). أظهر الإنزيم الذي تم التعبير عنه في بكتيريا ايشريشياكولاي كمية معنوية من النشاطية. تمت تنقيه الكولين كايناز الذي تم التعبير عنه جزئيا على عمود نيكل–نيترايلو ثلاثي حمض الخليك ودرست خصائصه. أظهر الكولين كايناز المستنسخ المنقى جزئيا احتياجه إلى ثلاثي إدينوسين الفوسفات والمغنيسيوم. كما كان الرقم الهيدروجيني المثالي في المدى ٧,٥ – ٩,٥، وكان ثابت ميكائيلس (K_m) للكولين هو 6.4×10^{-4} مولار. تم اختبار المثبطات المحتملة المختلفة ولكن الأورثو فينانثرين فقط كان له ذلك التأثير المعنوي. كما تم قياس مستوى التعبير عن جين الكولين كايناز في الأوراق والجذور والطلائع في المراحل العمرية للبازلاء (٤، ١٥، ٣٠ يوما) باستخدام تفاعل السلسلة المبلمر ذو الوقت الحقيقي (Real-time PCR) حيث ظهر أن مستوى التعبير متشابه بين الأنسجة ولا يختلف كثيرا في المراحل العمرية للنبات.