



Arginine kinase of the sheep blowfly *Lucilia cuprina*: Gene identification and characterization of the native and recombinant enzyme

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ABSTRACT

Arginine kinase (ATP:L-arginine ω -N-phosphotransferase, EC2.7.3.3.; AK) is an enzyme with crucial functions in the energy metabolism of insects and other invertebrates as well as some protozoa and has been proposed as a parasitocidal and pesticidal drug target. In this study we report the identification, cDNA cloning, genomic gene structure and functional expression of an AK gene from the parasitic sheep blowfly *Lucilia (L.) cuprina*. The blowfly AK-encoding gene is devoid of introns and present in a single copy in the *L. cuprina* genome. AK is present in *L. cuprina* flies as a highly expressed soluble enzyme and is more abundant in the thorax, where it represents up to 2% of the total soluble protein, compared to head or abdomen. Guanidino substrate specificity studies show that *L. cuprina* AK is stereospecific for the L-form over the D-form of its specific substrate arginine. Furthermore, the presence of a free or esterified carboxylic group as well as an unsubstituted α -amine group are essential for acceptance of the L-arginine substrate while modifications in the aliphatic side chain are better tolerated. The apparent Michaelis–Menten constants K_M and the molecular size of the recombinant enzyme are virtually identical to that of the native enzyme suggesting that the gene cloned in this study encodes the highly expressed AK enzyme of the sheep blowfly.

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1. Introduction

Phosphagen kinases form a large family of proteins that catalyze the reversible transfer of the γ phosphoryl group of adenosine triphosphate (ATP) to the guanidino groups of common cellular metabolites such as creatine and arginine (Fig. 1A), and of the less common guanidino metabolites glycocholine, taurocholine, ophe-line or lombricine [1]. The phosphorylated forms of these guanidino compounds are collectively called phosphagens. In conjunction with their cognate phosphagen kinases, they play key roles in cellular energy metabolism as ATP buffers. Further functions of phosphagen/phosphagen kinase systems, such as intracellular energy transport, regulation of inorganic phosphate concentration and proton buffering, are discussed [2–9].

In arthropods, phospho-L-arginine/arginine kinase appears to be the only existing phosphagen system [10], and has been demonstrated biochemically in various species of the chelicerata, crustacea and hexapoda [11–14]. In recent years, it has been discovered that arginine kinase of different arthropods acts as a major allergen affecting humans via food intake [15–18] or via environmental exposure [19–24].

Arginine kinase has been detected and investigated in a broad range of insects species [12,25–33]. The prominent role of arginine kinase in energy metabolism of insects as well as the fact that loss of function mutations in the corresponding *Drosophila melanogaster* gene proved to be lethal [34] have led to suggestions that this enzyme may be a molecular target for novel insecticides [30,33,35].

The sheep blowfly *Lucilia (L.) cuprina* is a major pest species of the sheep industry in many areas of the world. Disease is caused by infestations of sheep with the larvae of *L. cuprina*, a condition called myiasis [36]. Economic losses of the sheep industry due to myiasis have been estimated to be more than \$160 million annually in Australia alone [37,38]. Blowfly control has been attempted by general sheep management practises, selection of naturally resistant sheep, and vaccination approaches, but continues to rely mainly on insecticide use [36]. However, increasing numbers of reports on resistance development to various market products have led to considerable concern [39–44], and the development of new insecticidal compounds as additional blowfly strike prevention and treatment options is highly desirable.

In this study, we have identified and characterized the gene encoding arginine kinase in *L. cuprina*. We report the molecular cloning, the genomic structure and the heterologous functional expression of this blowfly enzyme. Furthermore we show data on the kinetic properties of the native and the recombinant arginine kinase as well as the solubility and distribution within the adult

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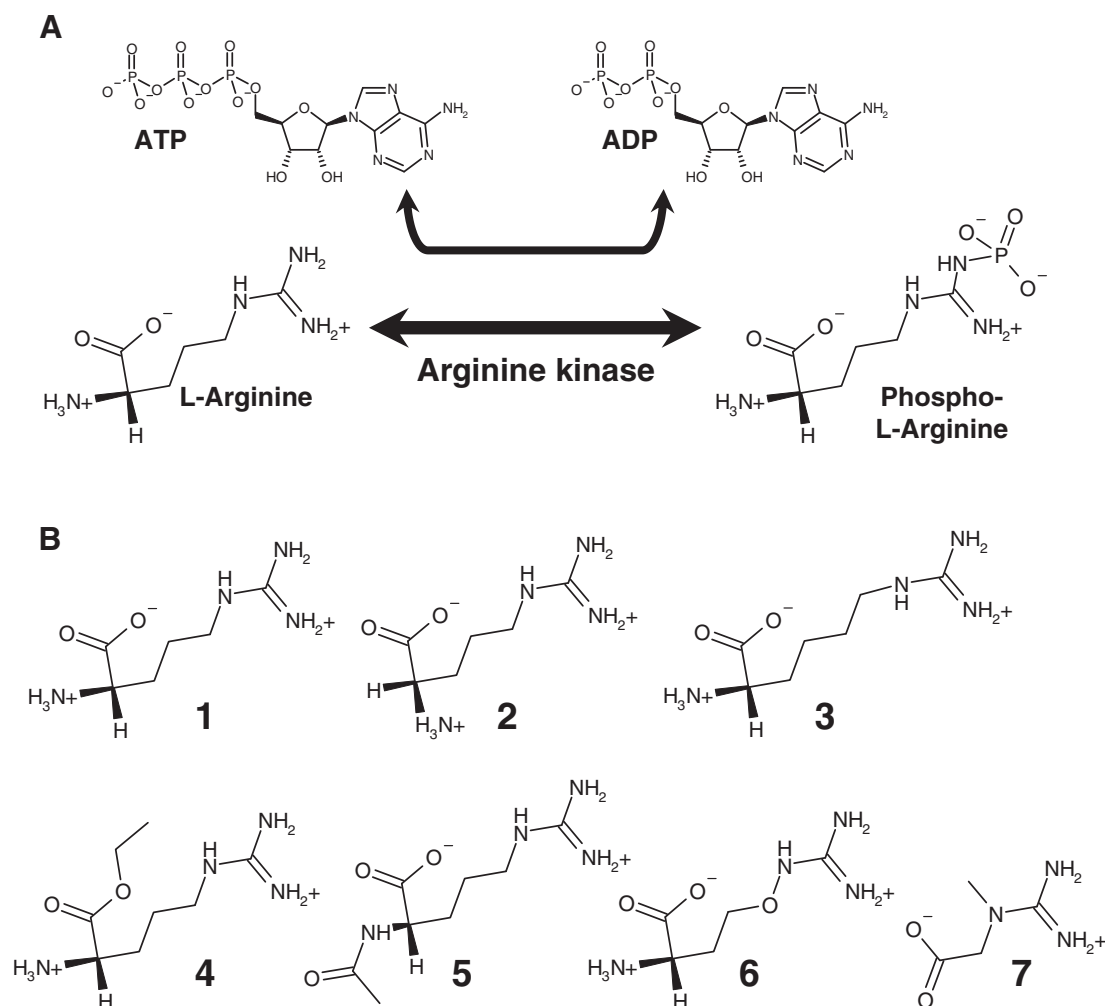


Fig. 1. Chemical structures of arginine kinase substrates. (A) Equation of the arginine kinase reaction. (B) Chemical structures of guanidino compounds used in arginine kinase assays. L-arginine (1), D-arginine (2), L-homo-arginine (3), L-arginine-*o*-ethyl ester (4), L-N α -acetyl-arginine (5), L-canavanine (6), and creatine (7).

blowfly. Finally, the potential of blowfly arginine kinase as an insecticidal target is discussed.

2. Materials and methods

2.1. Bacterial strains, plasmids, chemicals, column materials and insects

Bacterial cultures were grown in Luria–Bertani (LB) medium modified with supplements as specified. The cloning vector pCR2.1-Topo and its bacterial host strain *Escherichia* (*E.*) *coli* Top10 were from Invitrogen. The hexa-His tag expression vector pQE30 and its *E. coli* host strain M15 as well as Ni²⁺-NTA agarose were from Qiagen. L-arginine, D-arginine, L-homo-arginine, L-canavanine, L-arginine-*o*-ethyl ester, L-N α -acetyl-arginine, creatine, agmatine, phospho-L-arginine, glucose, glucose-6-phosphate dihydronicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP⁺), phosphoenolpyruvate, adenosine diphosphate (ADP), adenosine triphosphate (ATP), hexokinase, glucose-6-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase were from Sigma. *L. cuprina* adult flies used in this study were collected from long term cultures maintained at Intervet Innovation GmbH, Schwabenheim/FRG, Germany.

2.2. Identification, isolation and phylogenetic analysis of the *L. cuprina* arginine kinase gene

Total RNA was extracted from adult *L. cuprina* flies by a modification of the guanidinium thiocyanate/phenol extraction method ([45]; Trizol, Sigma). Reverse transcription (RT-) PCR was performed using the Titan one tube RT-PCR system (Roche) with total RNA (0.5–1 μ g/50 μ l) as template. In some cases, reverse transcription of total RNA was performed in a separate step followed by PCR under various conditions. For the identification of arginine kinase genes of *L. cuprina*, degenerate primer pairs were constructed from the peptide sequences DDHFLFKEG and EHT(E/K)(A/S)EGG, which are conserved in arthropod species as distant as *Limulus polyphemus* and *D. melanogaster* (Fig. 1). Degenerate primer RT-PCR was performed using total RNA isolated from *L. cuprina* imagines. RT-PCR products of the expected size (~400 bp) were cloned and sequenced. The deduced protein sequence of the degenerate primer RT-PCR products (416 bp) showed 92.6% sequence identity to the *D. melanogaster* arginine kinase gene (NM_079264). The missing cDNA sequences of the *L. cuprina* arginine kinase gene were then obtained by nested 5'-rapid amplification of cDNA ends (RACE; first specific primer CACCACGGGTACCGCGGACTTGCAAGTTG and second specific primer GGGACCTTGATGTGCACGGAGGCAC) and 3'-RACE (first specific primer GATCGTTTCTTGCAAGCCCAACGCCTGCC

and second specific primer CAACAAGACCTTCTTGGTCTGGTGAAT-GAGG) using total RNA from fly imagos as templates. 5'-RACE- and 3'-RACE-PCR was performed using the SMART RACE cDNA Amplification Kit (Clontech) as outlined by the manufacturer, using total RNA as template, *L. cuprina* arginine kinase specific 5'-RACE and 3'-RACE primers derived from specific degenerate primer RT-PCR products, and generic flanking 5'- and 3'-RACE primer. Based on the deduced start and stop codon positions in the 5'- and 3'-RACE product sequences, PCR primer (CTGGATCCACCATGGTTGATGCTGCTGTCTTGC and GACCCGGGTTACATGCTCTTTTCGATCTTGATG) were then designed for the RT-PCR amplification of the full length gene from 5'- and 3'-RACE cDNA, respectively, derived from fly total RNA. The restriction enzyme sites introduced by the primer are underlined. The PCR product was cloned into pCR2.1-Topo and sequenced. Thereby a 1129 bp PCR product with a 1071 bp open reading frame (*lcak*) encoding *L. cuprina* arginine kinase (LcAK) was identified. A consensus sequence devoid of PCR errors was identified by sequencing of three independent plasmid-cloned PCR fragments as well as direct PCR product sequencing and performing

ClustalW alignments with the translated insert DNA sequences. The *lcak* open reading frame was excised by Bam HI/Sma I digests and ligated into Bam HI/Sma I cut pQE30 (pQE30-*lcak*). For overexpression experiments pQE30-*lcak* was transformed into *E. coli* M15. ClustalW multiple sequence alignments of *L. cuprina* arginine kinase and other arthropod enzymes, the generation of phylogenetic trees and bootstrap analyses were done using the DNASTar Lasergene software package.

2.3. Protein expression in *E. coli* and protein purification

Overnight cultures of *E. coli* M15 carrying pQE-*lcak* in LB medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin (37 °C) were diluted 1:10 in fresh antibiotics-containing medium, cultured for 1 h at 37 °C and then cooled to room temperature. LcAK expression was induced by the addition of 1 mM isopropyl thio-β-D-galactoside (IPTG). After a further 4 h of room temperature culture, the cells were harvested and frozen at -80 °C. Purification of the hexa-His-tagged LcAK (=His₆-LcAK) was performed

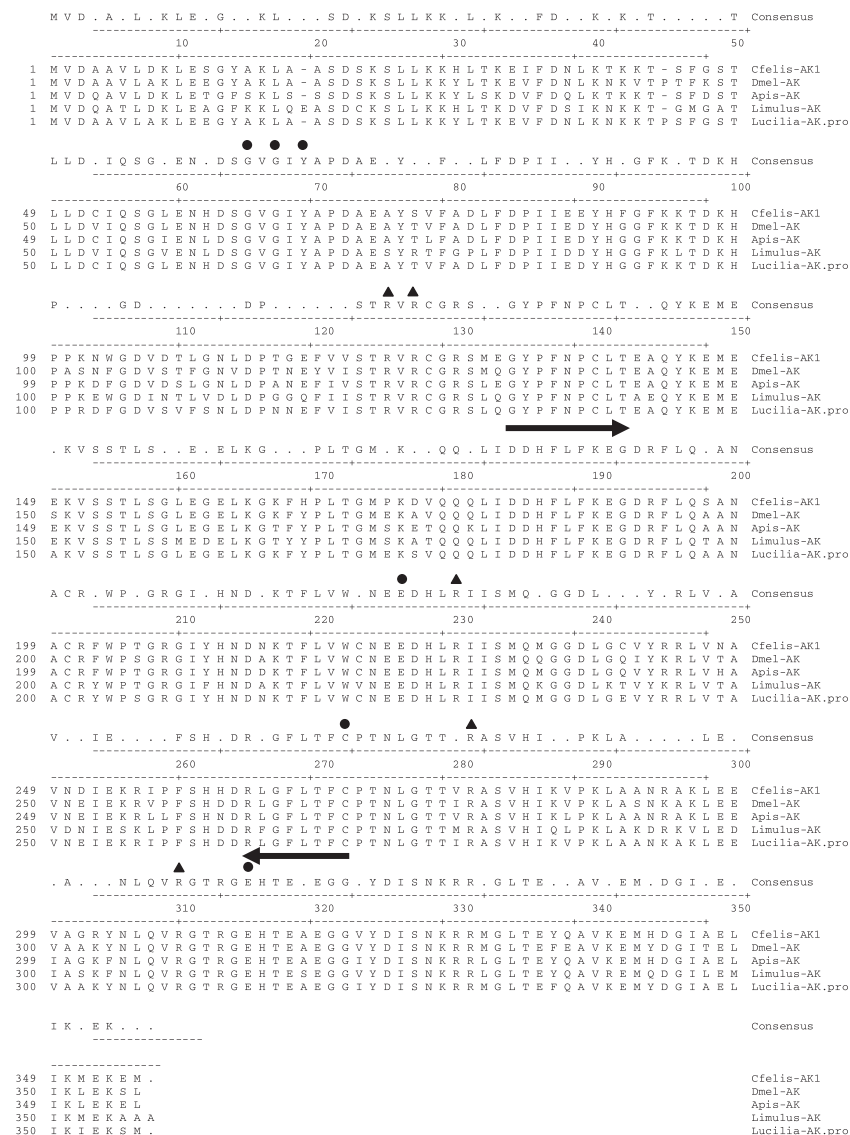


Fig. 2. Alignment of AK protein sequences of *L. cuprina* and other arthropods. *L. cuprina* AK identified in this study (JQ088101) was aligned with *Drosophila melanogaster* AK1 (NM_079264), *Ctenocephalides felis* AK1 (FN421431), *Apis mellifera* AK (AF023619) and *Limulus polyphemus* AK (U09809). The peptide sequences used for degenerate primer design are marked with horizontal arrows. The conserved residues implicated in substrate guanidinium binding are marked by filled circles; the five conserved arginine residues implicated in ADP binding are highlighted by black triangles [49]. The alignment was carried out using ClustalW/DNASTar.

by *E. coli* extraction and native Ni²⁺-NTA agarose chromatography as outlined by the manufacturer (Qiagen), except for supplementing the elution buffer with 1 mM dithiothreitol (DTT). Enzyme-containing 250 mM imidazole eluates were stored at 4 °C, because freezing led to precipitate formation and loss of enzymatic activity.

2.4. Analytical methods

Protein concentration in column eluates was determined by the Coomassie blue dye binding method using bovine serum albumin as a standard [46]. A standard arginine kinase assay was performed in the direction of phospho-L-arginine production at 22 °C in 0.5 ml or 1 ml volumes of 50 mM Tris-HCl pH 7.5, 20 mM MgCl₂, 0.9 mM ATP, 0.2 mM NADH, 2.5 mM phosphoenolpyruvate, 4 mM DTT, 2 units/ml pyruvate kinase and 1 unit/ml lactate dehydrogenase. After addition of the arginine kinase sample, the reaction was started with 5 mM L-arginine (final concentration, which is saturating for LucuAK). For assays using *L. cuprina* extracts, background rates without specific substrates were determined and subtracted from the extract reaction rates to obtain the specific rates. For the determination of the apparent Michaelis-Menten constant (K_M) for L-arginine and for L-arginine-*o*-ethyl ester, the concentrations of these amino acids were varied between 0.2 and 10 mM, and the initial reaction velocity was recorded. ATP at a 0.9 mM concentration is not saturating for LucuAK. However, higher concentrations lead to pronounced inhibition of pyruvate kinase in the coupled enzyme detection system [32]. Therefore, the K_M values determined by this assay setup are apparent K_M s valid only for these specific conditions. To determine the K_M values for phospho-L-arginine, the assay was performed into the direction of ATP production. In this case 50 mM Tris-HCl pH 7.5, 20 mM MgCl₂, 10 mM glucose, 2 mM nicotinamide adenine dinucleotide phosphate (NADP⁺), 4 mM DTT, 2 units/ml hexokinase and 1 unit/ml glucose-6-phosphate dehydrogenase were used as basic buffer. For the determination of phospho-L-arginine K_M values, this solution was supplemented with 5 mM ADP (saturating concentration for LucuAK), and the phospho-L-arginine was varied between 0.05 and 5 mM. A standard acetylcholinesterase assay [47] was per-

formed at 22 °C in 1 ml volumes of 10 mM Na₂HPO₄/NaH₂PO₄ pH 7.4, 137 mM NaCl, 2.7 mM KCl (PBS) containing 0.5 mM DTNB. After addition of the acetylcholinesterase sample, the reaction was started with 1 mM AcTC (final concentration) and the progress of the reaction was recorded by measuring generation of 2-nitro-5-thio-benzoic acid via its absorption at 410 nm. Glucose-6-phosphate dehydrogenase was measured in 100 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 5 mM NADP⁺. After the addition of an enzyme-containing sample, Glucose-6-phosphate was added to 20 mM final concentration and the resulting reaction rate was determined by monitoring the increase in absorbance at 340 nm. SDS-polyacrylamide gel electrophoresis of purified proteins or extracts was performed using 10% acrylamide/bis-acrylamide (29:1) gels, that were subsequently either stained with Coomassie Blue or blotted onto nitrocellulose membranes for immunodetection of arginine kinase.

2.5. Isolation of *L. cuprina* arginine kinase genomic gene copies and Southern blot analysis

Genomic DNA from adult *L. cuprina* was isolated as outlined in standard protocols [48]. PCR (Expand High Fidelity PCR system, Roche) was performed to amplify genomic gene copies using genomic DNA (0.5 µg/50 µl) as template with the same primer combinations as for *lcak* full length cDNA amplification. Southern blot analysis of restriction enzyme-digested *L. cuprina* genomic DNA was performed by capillary blotting using cationized nylon membranes (Roche) as described in standard protocols [48]. Digoxigenin (DIG) labeled full length cDNA probes were generated by PCR with the PCR DIG probe synthesis kit (Roche) using the appropriate *lcak* cDNA or genomic DNA templates and the primer combination CTGGATCCACCATGGTTGATGCTGCTGTTCTTGC and GACCCGGGTTA CATGCTCTTTTCGATCTTGATG. Hybridization and detection of membrane-bound DNA probes was done according to the instructions of the digoxigenin hybridization and detection kit (Roche) using CDP-Star (Roche) as chemiluminescence substrate and corresponding films (Hyperfilm ECL; Amersham/GE) for exposure and development.

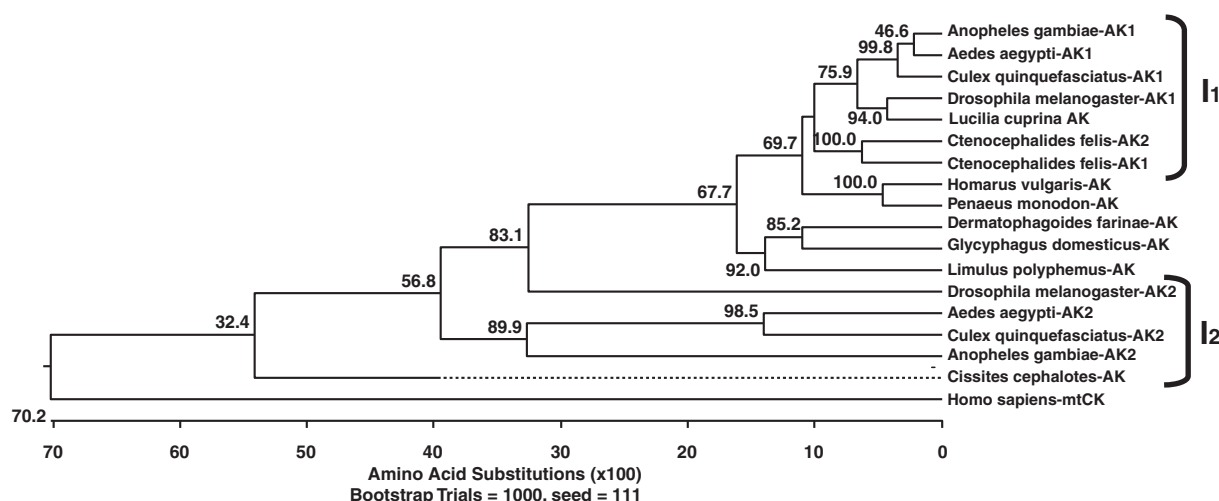


Fig. 3. Phylogenetic dendrogram of 16 arthropod AKs and one creatine kinase. The dendrogram (DNASTar) was derived from ClustalW-aligned protein sequences of *L. cuprina* AK identified in this study (JQ088101) together with 16 other arthropod AKs and one creatine kinase (CK) with the following Genbank accession numbers: *D. melanogaster* AK1 (NM_079264), *D. melanogaster* AK2 (BT012434), *Anopheles gambiae* AK1 (XM_001688645), *Anopheles gambiae* AK2 (XM_321020), *Aedes aegypti* AK1 (XM_001653673), *Aedes aegypti* AK2 (XM_001657339), *Culex quinquefasciatus* AK1 (XM_001849602), *Culex quinquefasciatus* AK2 (XM_001843516), *Ctenocephalides felis* AK1 (FN421431), *Ctenocephalides felis* AK2 (FN421427), *Homarus vulgaris* AK (AM940133), *Penaeus monodon* AK (AF479772), *Dermatophagoides farinae* AK (AY211951), *Glycyphagus domesticus* AK (EU106620), *Limulus polyphemus* AK (U09809) *Cissites cephalotes* AK (AB275000) and *Homo sapiens* mitochondrial CK (J04469). Bootstrap values (1000 replications) are shown at the branching points. The two insect AK groups proposed by Tanaka et al. [31] are indicated by the parentheses marked I1 (group 1 insect AKs) and I2 (group 2 insect AKs).

2.6. Analysis of arginine kinase in *L. cuprina* extracts by enzyme assays and immunoblot studies

For the analysis of arginine kinase solubility, 25 *L. cuprina* flies were homogenized in 10 ml ice-cold PBS, 4 mM dithiothreitol (DTT) using mortar and pestle followed by use of an Ultraturrax. After ultracentrifugation (100,000g at 4 °C for 30 min) the supernatant (SN1) was stored and the homogenization and centrifugation step was repeated once yielding SN2. The resulting pellet (P) was resuspended in 10 ml PBS, 4 mM DTT, 0.5% CHAPS. Samples of homogenate, SN1, and P were then analyzed by immunoblotting and standard arginine kinase assays. For immunoblots, 3 µl aliquots of the samples were mixed with 7 µl H₂O and 2.5 µl sample buffer (250 mM Tris–HCl pH 6.8, 10% (w/v) SDS, 500 mM DTT, 0.1% (w/v) bromophenol blue), heated to 95 °C for 5 min, loaded onto 10% SDS–polyacrylamide gels together with a 20 ng standards of recombinant His₆-LcAK. The electrophoretically separated proteins were electrotransferred (Transblot, BioRad) to a nitrocellulose membrane (Protean, Whatman). After blocking of non-specific binding sites with 5% skim milk powder in phosphate-buffered saline (PBS, 10 mM Na₂HPO₄/NaH₂PO₄ pH 7.4, 137 mM NaCl, 2.7 mM KCl) for 1 h at room temperature, the membrane was incubated overnight at 4 °C in 5% skim milk powder/PBS containing affinity-purified rabbit anti-DmAK antibodies [32] corresponding to an approximately 1:20 dilution of the initial antiserum. The membrane was washed four times with PBS, 0.1% Tween-20 followed by a 30 min incubation in a 1:20,000 dilution of horseradish peroxidase-coupled goat anti-rabbit IgG (Jackson ImmunoResearch) in 5% skim milk powder/PBS. After five washes with PBS, 0.1% Tween-20, bound antibody conjugates were detected using a peroxidase chemiluminescence substrate (ECL, Amersham) using films (Hyperfilm ECL, Amersham/GE) as outlined by the manufacturer.

3. Results

3.1. Identification and isolation of the *L. cuprina* arginine kinase gene *lcak*

For the identification of the arginine kinase gene of *L. cuprina*, highly conserved peptide sequences present in arginine kinases from diverse insect species such as *Drosophila* (*D.*) *melanogaster*, *Ctenocephalides* (*C.*) *felis* and *Apis* (*A.*) *mellifera* as well as from the horseshoe crab *Limulus* (*L.*) *polyphemus* were determined (Fig. 2). Degenerate primer pairs from several peptide sequences were constructed and degenerate primer RT-PCR was performed using total RNA isolated from *L. cuprina* adult flies. Degenerate primer pairs derived from DDHFLFKKEG and EHT(E/K)(A/S)EGG (Fig. 2) gave rise to a RT-PCR product of the expected size (~416 bp), which was cloned and sequenced (submitted to GenBank, JQ088100). The deduced protein sequence of the degenerate primer RT-PCR product showed 92.8% identity to the corresponding region of the *D. melanogaster* arginine kinase gene (NM_079264). The cDNA sequence of the *L. cuprina* arginine kinase gene was then completed by 5'- and 3'-RACE using total RNA as template (submitted to GenBank, JQ088101). Based on the deduced start and stop codon positions in the 5'- and 3'-RACE product sequences, PCR primers were then designed for the RT-PCR amplification of the full length gene from total RNA. A RT-PCR product with a 1071 bp open reading frame (termed *L. cuprina* arginine kinase, *lcak*) was identified (JQ088101). Consensus sequences devoid of PCR errors were identified by sequencing of three independent plasmid-cloned PCR fragments and by direct sequencing of the PCR product. The deduced polypeptide sequence of this new *L. cuprina* gene, LcAK, showed extensive sequence identity to arginine kinases from other species, particularly to *D. melanogaster* arginine kinase (92.1%), but

also to *C. felis* arginine kinase 1 (88.8%) and *L. polyphemus* arginine kinase (74.7%) (Fig. 2). Inspection of the LcAK peptide sequence showed that, six out of seven amino acid residues that were reported to interact with the substrate arginine in *L. polyphemus* AK (G64, G66, Y68, C271, E314, E225; [49]) and all five arginines reported to interact with the ADP phosphates (R124, R126, R229, R280, R309; [49]) were conserved (Fig. 2). Phylogenetic analysis, as illustrated in the DNASTar/ClustalW-based molecular tree (Fig. 3) showed that the *lcak* gene identified and cloned in this study belongs to the arginine kinase family, and more specifically to the insect group 1 arginine kinases [31] (11, Fig. 3).

3.2. Genomic organization and copy number of the *L. cuprina* arginine kinase gene *lcak*

For the isolation of a genomic copy of *lcak*, PCR was performed using the respective full length primer and *L. cuprina* genomic DNA as template. The resulting PCR product was subsequently cloned and sequenced. Inspection of the resulting sequences showed that the genomic copy of *lcak* is devoid of introns (submitted to GenBank, JQ088102). To investigate the copy number of *lcak* in the *L. cuprina* genome, Southern blot analysis was performed using a digoxigenin-labeled hybridization probe encompassing the entire

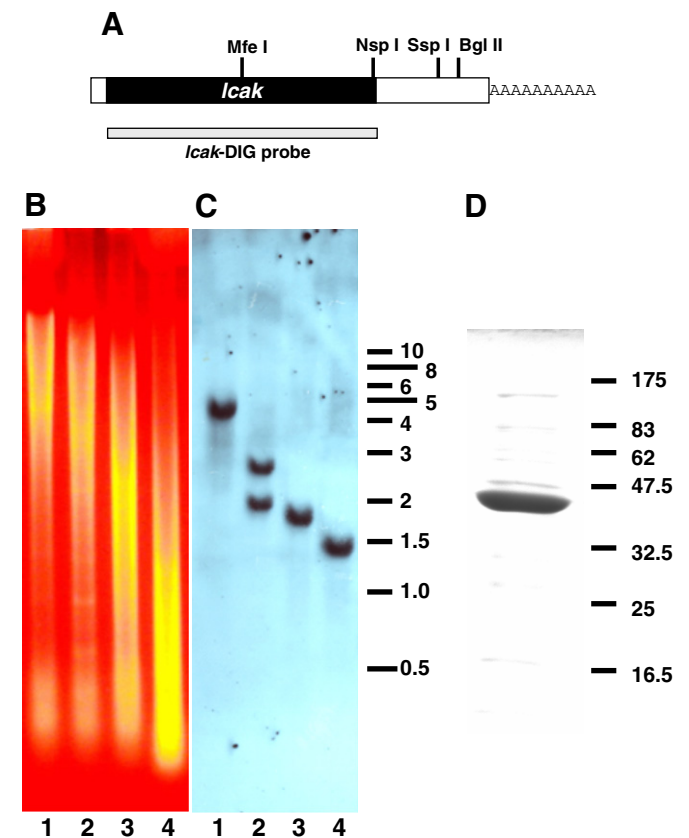


Fig. 4. Gene structure and Southern Blot analysis of the *L. cuprina* arginine kinase gene *lcak*, and SDS-PAGE of *E. coli*-expressed *lcak*. (A) Organization of an *lcak*-derived mRNA-derived cDNA compiled from 5'-RACE product, 3'-RACE product and the full length cDNA open reading frame (black area), the 5'- and 3'-untranslated regions (UTRs) are in white, the 3'-polyA tail is indicated. The sequence encompassing the DIG-labeled Southern blot probes *lcak*-DIG is indicated by the gray bar. (B) ethidium bromide-stained 1% agarose gel of restriction enzyme digests of *L. cuprina* genomic DNA. (C) Southern blot membrane derived from (B) probed with the DIG-labeled probe *lcak*-DIG. (B and C) The individual lanes were loaded with 10 µg of *L. cuprina* genomic DNA digested with the following restriction enzymes: lanes 1, Bgl II; lanes 2, Mfe I; lanes 3, Nsp I; lanes 4, Ssp I. The positions of DNA fragments from a 1 kb ladder (NEB) are indicated in kb. (D) 12.5% SDS-PAGE of 10 µg His₆-LcAK. The relative positions of protein size markers are indicated in kDa.

Table 1

L. cuprina arginine kinase: determination of apparent Michaelis–Menten constants K_M for arginine substrates.

	K_M of <i>L. cuprina</i> AK, UC supernatant (μM)	K_M of <i>E. coli</i> -expressed His ₆ - <i>L. cuprina</i> AK (μM)
L-arginine	594	603
L-phospho-arginine	1166	1432
L-o-ethyl-arginine	n.d.	2796

n.d.: Not determined.

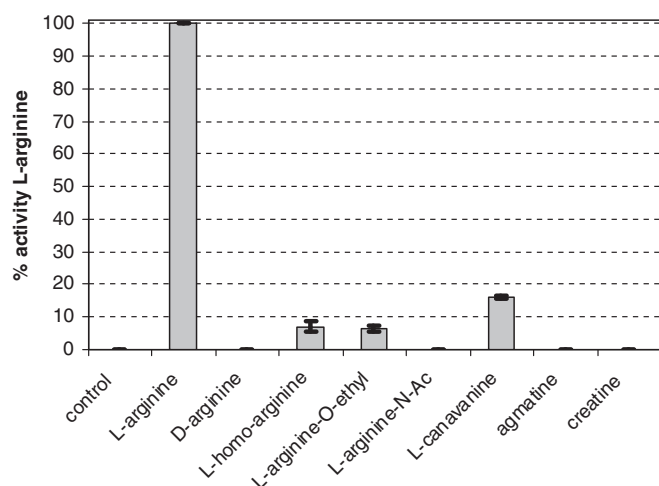


Fig. 5. Guanidino substrate specificity of His₆-LcAK – standard arginine kinase conditions were applied and the reaction rates at 5 mM guanidino substrate concentrations were determined. The standard deviation of triplicates is indicated.

open reading frame (Fig. 4A). For the digestion of *L. cuprina* genomic DNA, two restriction enzymes were chosen that cut once in the 3'-untranslated region downstream of *lcak* but not within its open reading frame, one enzyme that cuts immediately adjacent to the stop codon, and, additionally, an enzyme that cuts once in the middle of the *lcak* open reading frame (Fig. 4A). The hybridization signal patterns observed were consistent with a single copy gene situation for *lcak* in the genome of *L. cuprina* (Fig. 4B and C).

3.3. Heterologous expression of *L. cuprina* arginine kinase LcAK, and characterization of the purified enzyme

The PCR primer-introduced flanking Bam HI and Sma I restriction enzyme sites were used to subclone the full length *lcak* gene of *L. cuprina* into the *E. coli* expression vector pQE30. Protein expression was performed in *E. coli* M15 at room temperature, which yielded largely soluble expression products that were purified by Ni²⁺-NTA agarose chromatography via their N-terminal His₆-tags. The expressed gene product of *lcak* showed the expected size of 41.5 kDa on Coomassie Blue-stained SDS–polyacrylamide gels with an estimated purity greater than 90% (Fig. 4D). The yields were in the range of 2 mg/100 ml *E. coli* culture and the resulting recombinant protein showed enzymatic activity in a standard arginine kinase assay (phospho-L-arginine production), with a specific activity up to 16 units/mg. The determination of the apparent Michaelis–Menten (K_M) constants for the arginine kinase substrates L-arginine and phospho-L-arginine gave values in the high μM /low mM range, that are expected for this type of enzyme (Table 1). The investigation of enzyme stereospecificity for the chiral arginine substrate showed a high specificity of LcAK for L-arginine over D-arginine (Figs. 1B and 5). Among the other potential guanidino substrates (Fig. 1B) investigated, only L-canavanine showed an appreciable reaction rate (~16% of that obtained with L-arginine), while L-arginine-o-ethyl ester and L-homo-arginine were poor substrates with only 6% and 7% of the L-arginine reaction rate, respectively. L-N α -acetyl-arginine, agmatine and creatine were no substrates for LcAK (Fig. 5).

3.4. Characterization of arginine kinase from *L. cuprina* extracts

To investigate whether arginine kinase is a soluble enzyme, *L. cuprina* adult flies were homogenized in PBS and subjected to ultracentrifugation. Determination of arginine kinase in the ultracentrifugation supernatant and the resuspended pellet showed that >99.7% of the enzymatic activity resided in the soluble supernatant fraction (Fig. 6A). The solubility of AK was confirmed by an immunoblot with affinity-purified arginine kinase antibodies: in total fly lysates generated without addition of detergent, a single band was observed at a position consistent with the expected size of the *lcak* gene product (~40 kDa). After ultracentrifugation of the lysate, the immunoreactive protein was present quantitatively in the superna-

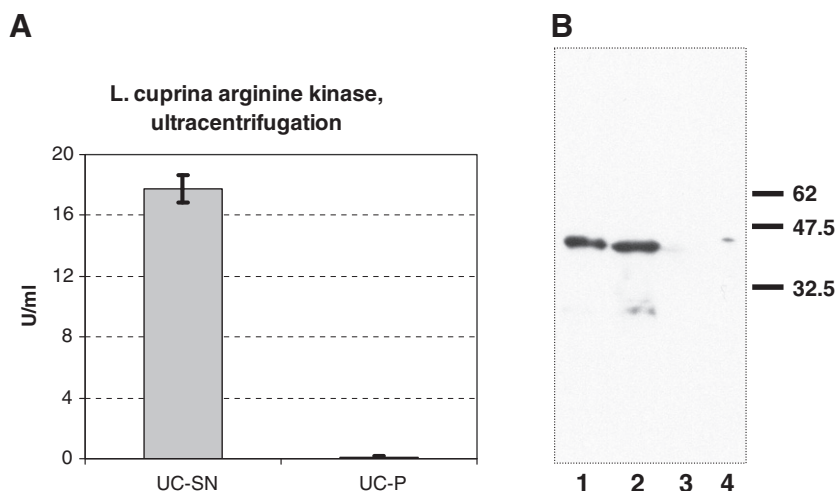


Fig. 6. Arginine kinase assay and 12.5% SDS–PAGE/immunoblot analysis of ultracentrifugation fractions from *L. cuprina* fly homogenates. (A) Determination of arginine kinase activity in *L. cuprina* fly homogenate ultracentrifugation supernatant (UC-SN) and the corresponding reconstituted pellet (UC-P) by a standard assay with 5 mM L-arginine. The standard deviation of triplicates is indicated. (B) Immunoblot analysis of *L. cuprina* adult fly homogenate (lane 1), ultracentrifugation supernatant (lane 2) and ultracentrifugation pellet (lane 3) using affinity-purified rabbit *D. melanogaster* AK1 antibodies. Recombinant His₆-LcAK (20 ng) was loaded as a positive control (lane 4). The relative positions of protein size markers are indicated in kDa.

tant, consistent with the enzymatic activity data (Fig. 6B). This result also suggests that little, if any, AK is trapped in vesicular compartments such as mitochondria. Determination of the Michaelis–Menten constants K_M of the arginine kinase from the ultracentrifugation supernatant for the substrates L-arginine and L-phospho-arginine gave values very close to those obtained for the recombinant enzyme (Table 1). Extraction of dissected body parts from adult flies and determination of specific arginine kinase activities showed an enrichment of the enzyme by approximately a factor three in the thorax relative to head and abdomen (Fig. 7A). To compare this AK distribution with those of a neural marker enzyme and an enzyme of the pentose phosphate pathway, we performed acetylcholinesterase and glucose-6-phosphate dehydrogenase assays. Here, the same extracts showed a pronounced enrichment of acetylcholinesterase in the head relative to thorax (factor 37) and abdomen (factor 146) (Fig. 7B), whereas glucose-6-phosphate dehydrogenase was enriched in the abdomen relative to head (factor 3) and thorax (factor 10) (Fig. 7C).

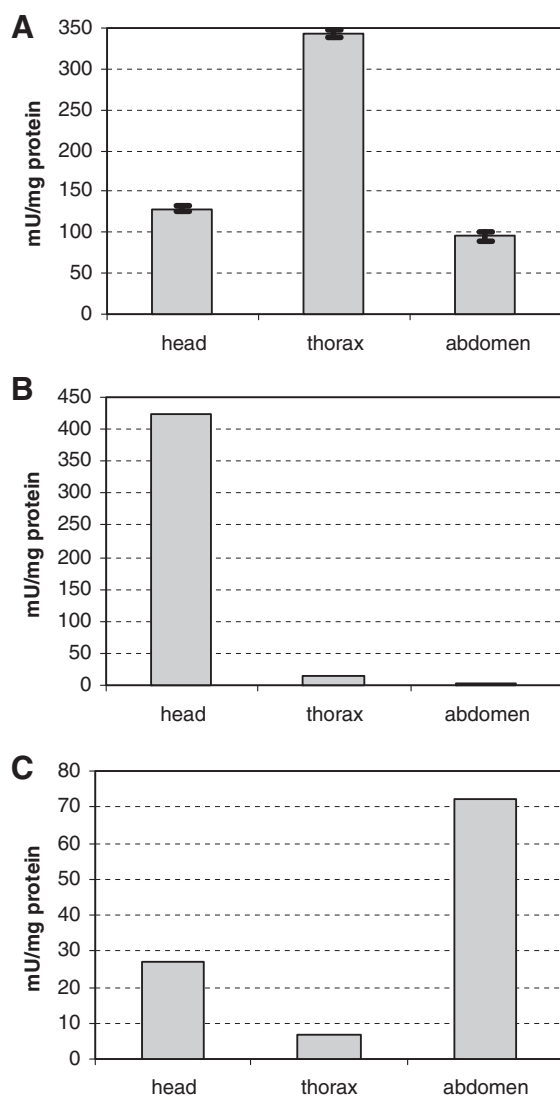


Fig. 7. Distribution of specific enzyme activities in body parts of dissected *L. cuprina* flies. (A) Arginine kinase activity. (B) Acetylcholinesterase activity. (C) Glucose-6-phosphate dehydrogenase activity. The standard deviation of triplicates is indicated.

4. Discussion

Arginine kinase (AK) is an enzyme of crucial importance for the energy metabolism of insects [12,27,50,51], and is responsible for the high concentrations of L-phospho-arginine found in insect tissues [32,51–53]. In this study, we have identified the gene encoding AK in the pest insect species *L. cuprina*, the sheep blowfly (*lcak*). Full length cloning and sequence comparisons show that *L. cuprina* AK belongs to the group 1 AK subfamily of phosphagen kinases, distinct from a second subfamily (group 2) of putative insect AKs identified by bioinformatical methods [8].

Heterologous expression of *lcak* in *E. coli* and determination of enzymatic activity in the purified product show that this gene indeed encodes an active AK. The specific activity of the purified recombinant product (~16 U/mg) compares well to that reported for an AK purified from another cyclorrhaphan fly species, *Musca domestica* (10.28 U/mg, [26]). Investigation of recombinant *L. cuprina* AK guanidino substrate specificity shows that this enzyme does not accept creatine and is highly stereospecific for L-arginine versus D-arginine, different from an AK activity reported recently from an annelid worm [54]. Furthermore, L-arginine ceases to be a substrate for *L. cuprina* AK upon N α -acetylation and upon decarboxylation to agmatine, while esterification of the carboxyl group is tolerated with some loss of activity. These results indicate that while the carboxyl group appears to be crucial for AK substrate properties, its negative charge is not. Replacement of the terminal methylene moiety by oxygen (L-canavanine) and insertion of an additional methylene moiety (L-homo-arginine) also does not lead to complete loss of substrate property in *L. cuprina* AK. This suggests some plasticity of the *L. cuprina* AK guanidino substrate cavity.

The predicted molecular mass of ~40 kDa for the *lcak* gene product compares well with the size of the single band obtained in an immunoblot experiment with *L. cuprina* fly lysate and affinity-purified rabbit AK antibodies. Furthermore, the apparent Michaelis–Menten constants for the guanidino substrates L-arginine and phospho-L-arginine for blowfly-derived AK and for the recombinant enzyme are very similar. In *L. cuprina*, *lcak* is present in a single copy and is devoid of introns, reminiscent of the situation in *D. melanogaster* [8]. There is no evidence for the presence of N-terminal endoplasmic reticulum or mitochondrial import sequences, which together with the absence of predicted transmembrane regions suggests that *L. cuprina* AK is most likely a cytosolic enzyme. In agreement with this notion, *L. cuprina* AK is quantitatively recovered in the ultracentrifugation supernatant of blowfly homogenates, while the membrane- and organell-containing pellet is devoid of both AK enzyme activity and AK immunoreactivity. Close association of arthropod AKs with membranes has never been reported. AK present in insect mitochondria has been demonstrated for *Manduca sexta* [55], but in *D. melanogaster* [56], a cyclorrhaphan fly like *L. cuprina*, as well as in *Locusta migratoria* [51], AK appears to be exclusively cytosolic. In *L. cuprina* blowflies, AK is enriched in the thorax relative to head and abdomen, and may constitute up to 2% of the soluble protein in this body part of the insect. This enrichment is most likely not an extraction artifact, since the neural marker acetylcholinesterase [57] and glucose-6-phosphate dehydrogenase expected to be enriched in the fat body of the abdomen show the expected distribution pattern. The high AK concentration in the blowfly thorax may be due to the flight muscles present in this insect body part. It has been reported previously that locust flight muscles are a rich source of AK [51].

The exclusive phosphagen system of vertebrates is phosphocreatine/creatine kinase, and representatives of this enzyme, while belonging to the phosphagen kinase superfamily, show some differences to invertebrate and protozoal arginine kinases [7]. This led several authors to propose AK as a potential anti-parasite

chemotherapeutic target [5,58,59], and as a potential pesticidal molecular target [30,33,35]. In this respect, the lethal phenotype of *D. melanogaster* *ak1* gene mutants [34] appears to confirm the latter notion and to validate the potential pesticide target AK, but it should be considered that lethality may reflect a developmental phenotype. Conditional (e.g. temperature-sensitive) *D. melanogaster* *ak1* mutants may be more instructive in this respect, as a lethal phenotype under restrictive conditions in the adult insect may be the guidance for the action of an AK inhibitor. However, such mutants have not yet been reported. Another potential disadvantage of AK as an insecticidal target is the high abundance of the enzyme, at least in *L. cuprina* (this study) and in the cat flea *Ctenocephalides felis* [32], but most likely also in other insects. This could make the identification of inhibitory compounds with sufficient potency as well as the application of these compounds under field conditions a major challenge. Although some compounds interfering with AK activity have been identified [35,60], no outstanding AK inhibitors have been found yet.

The broad occurrence of AK in many disease-causing protozoa and invertebrates as well as pest insects, and its absence in mammals is, however, a clear asset of this molecular target. With respect to potential screening efforts, the enzymatic assay of AK is easily adaptable to high throughput screening technology [61], which should make the identification of potent inhibitors a straightforward task.

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