

A novel splicing variant encoding putative catalytic α subunit of maize protein kinase CK2

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Abstract

A cDNA highly homologous to the known catalytic α subunit of protein kinase CK2 was cloned from maize (*Zea mays*). It was designated *ZmCK2 α -4* (accession no. AAF76187). Sequence analysis shows that *ZmCK2 α -4* and the previously identified *ZmCK2 α -1* (accession no. X61387) are transcribed from the same gene, *ZmPKCK2AL* (accession no. Y11649), but at different levels in various maize organs and at different stages of development. The cDNA encoding *ZmCK2 α -4* has three potential translation initiation sites. The three putative variants of *ZmCK2 α -4* were expressed in *Escherichia coli* as glutathione S-transferase (GST)-fusion proteins and purified from bacterial extracts. In contrast to the previously characterized *ZmCK2 α s*, the obtained GST:*ZmCK2 α -4* proteins were catalytically inactive as monomers or in the presence of equimolar amounts of the human CK2 β . However, GST:*ZmCK2 α -4* did phosphorylate casein in the presence of a large excess of the β subunit. The activity of *ZmCK2 α -4* toward casein could also be stimulated by increasing ATP concentration. Modeling studies have shown that there is no interaction between the N-terminal segment of *ZmCK2 α -4* and the activation loop responsible for constitutive catalytic activity of CK2 α . Preliminary results suggest that *ZmCK2 α -4* may function as a negative regulator of other CK2s, and at certain circumstances as a holoenzyme which catalytic activity is stimulated by specific regulatory subunit(s).

Abbreviations: AS, alternative splicing; AFEs, alternative first exons; CK2, caseine kinase 2.

Introduction

Protein kinase CK2 is a serine/threonine protein kinase present in all eukaryotic cells. In contrast to other protein kinases, CK2 is not regulated by second messengers such as calcium or lipids, but is active constitutively and it can use both ATP and GTP as phosphate donors. Over 300 proteins are known to be substrates of CK2 (for review see Litchfield 2003; Meggio and Pinna 2003). This large number of proteins with essential biological functions that interacts with and are phosphorylated by CK2 supports the idea that the kinase is involved in numerous cellular processes such as proliferation, apoptosis, differentiation, transformation, transcriptional control, signaling, and others (Guerra and Issinger 1999; Litchfield 2003; Bibby and Litchfield 2005). CK2 forms a stable heterotetramer, $\alpha_2\beta_2$, where α is the catalytic and β the regulatory subunit (Riera et al. 2001a; Litchfield 2003). The regulatory β subunit stimulates the activity of CK2 α (Grankowski et al. 1991), stabilizes the CK2 heterotetramer (Meggio et al. 1992) and provides specificity for the interaction with substrates and regulators (Bidwai et al. 1993; Litchfield 2003; Bibby and Litchfield 2005). Monomeric forms of CK2 comprising the catalytic α subunit have been identified in plants, including maize (Dobrowolska et al. 1992) and broccoli (Klimczak and Cashmore, 1994).

The current knowledge of CK2 comes mainly from studies on animal and yeast cells, and less is known about CK2 from plants. Studies concerning functions of plant CK2 have shown that this enzyme is involved in many processes, including light responses and growth control (Lee et al. 1999), cell division and cell cycle regulation (Espunya et al. 1999, 2005; Moreno-Romero et al. 2008), seed development (Ciceri et al. 1997), and salicylic acid (SA) (Hidalgo et al. 2001; Kang and Klessig, 2005) and abscisic acid (ABA) signaling pathways (Riera et al. 2004). CK2 interacts and phosphorylates many transcription factors, affecting their DNA binding activity (Klimczak et al. 1995; Sugano et al. 1998, 1999; Lee et al. 1999; Hardtke et al. 2000). Among these transcription factors, CCA1 (circadian clock-associated 1) and LHY (late elongated hypocotyl) are essential for the regulation of the endogenous circadian clock in *Arabidopsis* (Sugano et al. 1999). CK2 also participates in the response to osmotic stress in plants and yeast. Over-expression of the catalytic subunit of sugar beet CK2 (*BvCKA2*) in yeast increases yeast tolerance to NaCl (Kanhonou et al. 2001). Moreover, expression of *BvCKA2* is induced after salt treatment of sugar beet. In plants, genes encoding the α and β subunits belong to multigene families. In *Arabidopsis thaliana*, four genes encode CK2 α (Mizoguchi et al. 1993; Salinas et al. 2006) and four CK2 β (Collinge and Walker 1994; Sugano et al. 1998; Salinas et al. 2006). In *Zea mays*, until now, three cDNA clones encoding CK2 β and three cDNA clones encoding CK2 α (*ZmCK2 α -1*, *ZmCK2 α -2*, and *ZmCK2 α -3*) have been isolated and characterized (Dobrowolska et al. 1991; Peracchia et al. 1999; Riera et al. 2001a,b).

The present work reports the cloning of *ZmCK2 α -4*, a new, fourth isoform of CK2 α from maize. Genomic sequence analysis indicates that the *ZmCK2 α -4* cDNA is expressed from the same gene *ZmPKCK2AL* as the previously identified *ZmCK2 α -1*, and most probably expression of these two

transcripts is regulated by alternative splicing. We expressed ZmCK2 α -4 in bacteria and examined its enzymatic activity alone and in combination with human CK2 β . The transcript level of ZmCK2 α -1 and ZmCK2 α -4 was analyzed by RT-PCR in various maize organs at different stages of development.

Materials and methods

Cloning and sequencing of ZmCK2 α -4

A cDNA library (Stratagene, made from five-week maize leaves) was screened with ZmCK2 α -1 cDNA as probe. In total, about 200 000 plaques were transferred onto Hybond-N nylon filters, the filters were denatured for 7 min in 1.5 M NaCl, 0.5 M NaOH at room temperature, neutralized for 7 min in 1.5 M NaCl, 0.5 M Tris-HCl pH 8.0 and then washed for 10 min in 0.2 M Tris-HCl, pH 7.5. After washing, phage DNA was crosslinked to the membranes by UV irradiation and washed again in 0.2x SSC, 0.1% SDS at 55°C for one hour. For blocking, filters were pre-hybridized for 2 h at 42°C in 25% formamide, 5x Denhardt's solution (1x Denhardt's solution consists of 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), 5x SSPE, 0.1% SDS and 100 μ g/ml sonicated denatured salmon sperm DNA. Hybridization was performed with ³²P-labeled probes for 16 h at 42°C. Then the filters were washed twice for 30 min in 2 x SSC and 0.1% SDS. Dried filter was exposed to Amersham Hyperfilm MP for 2 days at -70°C with an intensifying screen. Twenty-eight phages showed very strong hybridization signal. Seven of them were subcloned into Bluescript plasmid (Stratagene) and then sequenced on an ABI3730 Genetic Analyzer (Applied Biosystems).

Expression of recombinant GST:CK2s

The pGEX-4T-2 expression vector system (Amersham Pharmacia Biotech) was used to express ZmCK2 α -4 in *E.coli*. Full-length cDNAs encoding ZmCK2 α -4 (variants I, II, and III) were amplified by PCR using the following primers:

5'-GTGGTGGGATCCATGCTTGCTATATCTGTGCTGTGC-3' (forward primer for variant I),
 5'-GTGGTGGGATCCATGACGATGATATATGCTACCGTCCG-3' (forward primer for variant II)
 or 5'-GTGGTGGGATCCATGATATATGCTACCGTCCG-3' (forward primer for variant III), and
 5'-TTTTCTTTTGC GGCCGCTCACGCTCGCGTCCTGCTGTT-3' (reverse primer for all three variants). The forward primers contained a restriction site for BamHI (underlined) and the START codon for appropriate variants. The reverse primer contained a restriction site for NotI (underlined) and the STOP codon common for the three variants. The PCR products were cloned into the pGEM[®]-T Easy vector (Promega) for amplification and subsequently subcloned into the BamHI/NotI sites of pGEX-4T-2 expression vector.

For biochemical characterization of ZmCK2 α -4 variants (I, II and III), known CK2 α (ZmCK2 α -1) was used as a reference. For expression of ZmCK2 α -1 in bacteria, two primers were designated to introduce a SmaI site (underlined) at the 5'-end

(5'-TATCCCCGGGTATGTCCAAGGCCAGGGTCTA-3'), and a Sall site (underlined) at the 3'-end (5'-AATGTTCGACTCACGCTCGCGTCCTGCTGTT-3') of the *ZmCK2 α -1* sequence. The PCR product was cloned into the pGEM-T Easy vector and subcloned into the SamI/SalI sites of pGEX-4T-1 expression vector. The three GST:ZmCK2 α -4 and GST:ZmCK2 α -1 constructs were transformed into *E. coli* BL21(DE3), expressed and purified using glutathione-Sepharose 4B resin according to manufacturer's instruction. For induction of GST:ZmCK2 α -1 and GST:ZmCK2 α -4 (variants I, II, and III) expression, 1 mM IPTG was added for 4 h at 37°C. To remove GST from the fusion proteins, overnight cleavage with thrombin was used. The treatment with thrombin was effective only in the case of ZmCK2 α -1. After purification, about 1.1 mg of the protein represented by a single band on SDS-PAGE (Fig. 4) was obtained from 1 liter of medium.

Purification of human CK2 β was performed as described by Sarno et al. (1996). About 1 mg of the purified protein represented by a single band on SDS-PAGE (Fig. 4) was obtained from 1 liter of medium.

Expression of ZmCK2 α -4 (variant III) using intein IMPACT[®] system in *E. coli*

For expression of the longest variant of ZmCK2 α -4 in bacteria, the intein IMPACT[®] system (New England Biolabs, USA) was used. Two primers were designated to introduce a NdeI site (underlined) at the 5'-end (5'-GTGGTGGGATCCCATATGCTTGCTATATCTGTGCTG-3') and a SpeI site (underlined) at the 3'-end

(5'-GTGGTGAACTAGTGCATCTCCCGTGATACACGCTCGCGTCCT-3') of the *ZmCK2 α -4* sequence. The PCR product was cloned into the pGEM[®]-T Easy vector and subcloned into the NdeI/SpeI sites of pTXB1 expression vector. ZmCK2 α -4 tagged with intein was transformed into *E. coli* ER2566 and expressed in medium with 0.1 mM IPTG for 15 h at 18°C. The protein was purified using chitin beads according to manufacturer's instruction.

Protein kinase assay in solution

The CK2 kinase in-solution assay was performed in a final volume of 50 μ l. The incubation mixture contained: 0.1 μ g of purified appropriate ZmCK2 α , 75 μ g of casein or 20 μ M peptide RRRDDDSDDD (Biosynton, German), 20 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 50 μ M γ [³²P]ATP (100-200 cpm pmol⁻¹). When CK2 β was present, 130 mM NaCl was added. After 20 min of incubation at 30°C, 40 μ l of the assay mixture with casein was spotted onto 3MM filter strip or the mixture with peptide onto P81 phosphocellulose, and then the filters were washed five times for 10 min in 5% TCA or in 0.3% H₃PO₄, respectively. Next, the filters were washed in 96% ethanol, dried, and the radioactivity was measured in a scintillation counter.

In-gel kinase activity assay

In-gel kinase assays were performed as described previously, with minor modifications (Szczegieliński et al. 2005). The recombinant proteins (ZmCK2 α -1, and three variants of ZmCK2 α -4) (0.1 μ g per lane) were separated on 12% SDS-polyacrylamide gel containing immobilized casein as substrate (0.5 mg/ml). After denaturation of the separated proteins with 6 M guanidine HCl followed by renaturation, the gel was incubated for 60 min at room temperature in 10 ml of buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM Ca(OAc)₂, 5 mM MgCl₂, 0.05 mM ATP supplemented with 50 μ Ci of γ [³²P]ATP). Unincorporated γ [³²P]ATP was removed by washing the gel with 5% TCA containing 1% sodium phosphate. Finally, the gel was stained with Coomassie Brilliant Blue, dried, and exposed to Amersham Hyperfilm MP (Amersham Pharmacia Biotech).

Molecular modeling

Model of ZmCK2 α -4 was built using Molecular Operating Environment (MOE, 2006.08) homology modeling approach. Briefly, protein model construction is based on a conventional homology modeling algorithm, exploiting Boltzmann weighted randomized modeling procedure adapted from Levitt, combined with specialized logic for the proper handling of insertions and deletions. Any selected atoms were included in energy tests and in minimization stages of the modelling procedure. The first step of the protocol is the alignment of the primary sequences of the two studied proteins; in our case ZmCK2 α -4 was built using ZmCK2 α -1 (PDB code: M2P) as a template. Protein alignment was performed using a BLOSUM 80 matrix without any alignment constraints. The selection of the final model selection was based on the best electrostatic solvation energy ranking, calculated using the generalized Born/Volume Integral (GB/VI) methodology (Labute 2007).

Plant material

Maize seeds (*Zea mays*, inbred line Rd 17-25), after overnight soaking in water at room temperature, were grown for 72 h at 26°C on wetted paper in the dark. The etiolated apical parts of the seedlings were harvested, immediately frozen in liquid nitrogen and stored at -80°C.

For leaves and roots, maize plants were cultivated hydroponically for 2 or 5 weeks in a growth chamber with a daily cycle of 16 h light (70-80 watt/m²) at 25°C and 8 h dark at 20°C.

RT-PCR analysis

Total RNA was isolated from seedlings, roots and leaves using TRI[®] Reagent (MRC, Cincinnati, OH) according to the protocol recommended by the manufacturer. cDNA was synthesized from 1 to 5 μ g of total RNA using HSRT 100 kit (Sigma). The reaction was performed for 50 min at 47°C in a final 20 μ l reaction volume containing 1 unit of enhanced avian reverse transcriptase, 500 μ M each dNTP, 1 U of RNase inhibitor, 3.5 μ M anchored oligo (dT) primer. Two μ l of the reverse transcription reaction was used for PCR in a 20 μ l volume containing 0.4 U of JumpStart AccuTaq LA DNA

polymerase, 100 μ M each dNTP, 1.5 mM MgCl₂, and 300 nM of appropriate primers. Routine PCR conditions were: 3 min, 94°C (first cycle), 30 s, 94°C; 30 s, 50-61°C; 1 min, 68°C (25-35 cycles), and 10 min, 68°C (final cycle). The PCR products were separated on 0.8% agarose gels and visualized by EtBr staining.

Specific forward primer for ZmCK2 α -1 based on 5' non-translated region sequence was 5'-GCGCCATGTCCAAGGCCAGGGTCT-3'. Specific forward primer for ZmCK2 α -4 based on 5' non-translated region sequence was 5'-CTGAGACCTCCCATGCTTGCTATA-3'. Reverse primer for ZmCK2 α -1 and ZmCK2 α -4 based on 3' non-translated region sequence was 5'-AGATGGTCTTCCGCTCTTTGCCTG-3'. As a control of the amount and quality of RNA, the transcript level of *actin* mRNA was monitored using primers: 5'-GCAGCTCGTAGCTCTTCTC-3' and 5'-AACAGGGAGAAGAGACCCA-3'.

Preparation of maize protein extract

Crude protein extract from maize was prepared as previously described (Szczegieliński et al. 2005).

Determination of protein concentration

Protein concentration was determined by the Bradford method (1979) using bovine albumin as a reference.

Accession Numbers

The accession numbers for the sequences described in this paper are as follows: *ZmPKCK2AL*, Y11649; ZmCK2 α -1, X61387; ZmCK2 α -2, Y11526; ZmCK2 α -3, AF239819; and ZmCK2 α -4, AAF76187.

Results

Molecular characterization of ZmCK2 α -4

Seven positive clones were isolated from a cDNA library from five-week old maize leaves using *ZmCK2 α -1* cDNA as probe. Sequence analysis of these clones revealed that six clones were identical with the sequence of *ZmCK2 α -1*. One cDNA clone encoding CK2 α (designated *ZmCK2 α -4*) was different from the previously cloned maize CK2 α s. *ZmCK2 α -4* was a full-length clone with an open reading frame beginning with three potential translation start codons and ending at a common stop codon. The predicted amino acid sequence of ZmCK2 α -4 exhibited very high homology with the previously known three catalytic α subunits of maize CK2: ZmCK2 α -1, ZmCK2 α -2, and ZmCK2 α -3 (Fig. 1). The highest identity shared with ZmCK2 α -1. The differences between ZmCK2 α -4 and ZmCK2 α -1-3 were located in the N-terminal region. A detailed analysis of all known sequences of maize CK2 α s has revealed that ZmCK2 α -4 and ZmCK2 α -1 originate from the same gene

ZmPKCK2AL, which was previously cloned and characterized (Peracchia et al. 1999). Most probably *ZmCK2 α -4* and *ZmCK2 α -1* are produced from the *ZmPKCK2AL* gene by alternative splicing (AS). Exon I is skipped in *ZmCK2 α -1*, whereas exon II is skipped in *ZmCK2 α -4*, without any disruption of the reading frame (Fig. 2 A). The first exon of *ZmCK2 α -4* starts from nucleotide 1034 and ends at nucleotide 1137, whereas the first exon of *ZmCK2 α -1* starts later, from 1663 and ends at nucleotide 1747 (Fig. 2 B). Apart from the first exon, the nucleotide sequences of *ZmCK2 α -4* and *ZmCK2 α -1* are identical. The N-terminal part of *ZmCK2 α -4* differs from those of all known CK2 α s from various organisms.

Expression and purification of *ZmCK2 α -4*

Three potential translation start sites were found in *ZmCK2 α -4* mRNA using the WEBGENE service program (<http://www.itb.cnr.it/sun/webgene/>) (Fig. 3). The most potent translation start site predicted by the program was that for Met II (variant II), but the other two (Met I and Met III) were also likely. To determine which of the three hypothetical variants of *ZmCK2 α -4* can be a functional protein, an *E. coli* expression system was used to produce fusion proteins with the GST tag. The three possible *ZmCK2 α -4* ORFs (I, II and III) were introduced into the bacterial expression vector pGEX4T-2. After induction with IPTG, the fusion proteins were isolated and purified using S-hexylglutathione resin as described in Materials and methods. About 0.4 mg of soluble proteins from 1 l of medium was obtained. Because approximately 90% of the recombinant proteins was present in an insoluble form as inclusion bodies, several conditions to obtain higher expression of soluble fusion proteins were tested. IPTG concentration, time course, and temperature were varied, and another bacterial strain Origami[®] was examined. The results of these experiments demonstrated that the duration and temperature of expression, concentration of IPTG, and the use of another *E. coli* strain essentially did not increase the solubility of the three GST:*ZmCK2 α -4* proteins (data not shown). Moreover, attempts to cleave GST from *ZmCK2 α -4* failed. Therefore, trials with the intein IMPACT[®] system for protein expression and purification were undertaken. The intein system utilizes the inducible self-cleavage activity to separate the target protein from the affinity tag (intein). The *ZmCK2 α -4* ORF (variant III) was introduced into the pTXB1 vector. Expression of the protein in *E. coli* was induced with IPTG and the protein was purified using chitin beads according to the manufacturer's instructions. However, a large amount of the insoluble protein was still present in inclusion bodies. The amount of soluble *ZmCK2 α -4* protein (Fig. 4) after purification using the InteIn IMPACT[®] system was about 20 μ g from 1 l of medium.

Biochemical and enzymatic characteristics of *ZmCK2 α -4*

The enzymatic activities of three variants of GST:*ZmCK2 α -4* (I, II, III) and *ZmCK2 α -1*, as a positive control, were analyzed by in-gel kinase assay with immobilized casein as substrate. Only *ZmCK2 α -1* was able to phosphorylate casein (Fig. 5 A). The three variants of *ZmCK2 α -4* were inactive. To verify

the results of the in-gel kinase assay, the activity of the three variants of GST:ZmCK2 α -4 (I, II, III), ZmCK2 α -4 III (without a tag, after expression using intein system), and ZmCK2 α -1 were tested in solution as described in Materials and methods. Casein or the specific peptide RRRDDDSDDD were used as substrates. All variants of GST:ZmCK2 α -4 and ZmCK2 α -4 III without a tag were inactive towards either substrate, whereas ZmCK2 α -1 was active towards both substrates (Fig. 5 B) (data for the peptide are not shown). To check whether the kinase is inactivated during the purification procedure, the activity of CK2 α s was also measured directly after expression in bacterial lysates of GST:ZmCK2 α -4 (I, II, III), ZmCK2 α -4-intein (variant III), and GST:ZmCK2 α -1. As controls the BL21 bacterial strains carrying pGEX4-2 expressing GST, and pTXB-1 expressing intein were used. After expression, cells were broken by sonication and the kinase activity was measured in solution using casein as substrate. In the total protein extract of the three variants of GST:ZmCK2 α -4 and ZmCK2 α -4-intein no enzymatic activity was detected, whereas the extract containing of GST:ZmCK2 α -1 was able to phosphorylate casein efficiently (data not shown).

The activity of the three variants of GST:ZmCK2 α -4, ZmCK2 α -4 (variant III) without a tag and ZmCK2 α -1 was also determined in the presence of regulatory β subunit. Human CK2 β in equimolar amounts with respect to CK2 α was able to stimulate the phosphorylation of casein by ZmCK2 α -1, in agreement with previously obtained results (Boldyreff et al. 1993). However, we did not observe a significant stimulatory effect of equimolar amounts of CK2 β on the activity of any variant of ZmCK2 α -4. Taking into consideration that the human CK2 β is not a physiological partner of ZmCK2 α -4, we tested the catalytic activity of GST:ZmCK2 α -4 (variant III) in the presence of an excess of human CK2 β . The obtained results indicated some activation of ZmCK2 α -4 towards casein in the presence of a large excess of CK2 β . CK2 β stimulated the activity of ZmCK2 α -4 in a dose-dependent manner (Fig. 5 C).

Considering that the activity of human CK2 α (mutated in its N-terminal part and defective in catalytic activity) could be partly restored by raising ATP concentration (Sarno et al. 2001), we tested the activity of GST:ZmCK2 α -4 (variant III) with various concentration of ATP (10-3000 μ M) using casein as substrate. The catalytic activity of ZmCK2 α -4, which is almost undetectable under standard assay conditions for human CK2 α and ZmCK2 α -1, was stimulated at high concentrations of ATP. The calculated K_m for ATP of ZmCK2 α -1 was 13 μ M (Niefind et al. 1999), whereas in the case of GST:ZmCK2 α -4 it was 1068 μ M (Fig. 6).

Homology modeling of ZmCK2 α -4 structure

ZmCK2 α -4 was modeled by homology basing on the crystal structure of ZmCK2 α -1 (Niefind et al. 1998). It had been reported earlier that the N-terminal segment of CK2 α , by interacting with the activation loop and a region close to it, is responsible for the constitutively active state of the kinase

(Sarno et al. 2002). The only differences between ZmCK2 α -4 and ZmCK2 α -1 occur in the N-terminal segment. By comparing the crystal structure of ZmCK2 α -1 and the obtained model structure of ZmCK2 α -4 we found major differences between these two proteins. In ZmCK2 α -1, the backbone amide group of Ala4 makes an important interaction with Tyr177 from the activation loop (Sarno et al. 2002). Instead of Ala4 present in ZmCK2 α -1, ZmCK2 α -4 has the more bulky Leu7 (Fig. 7). This leucine residue is in close contact with Tyr183 (corresponding to ZmCK2 α -1 Tyr177) and Tyr212 (about 1.88 and 1.9 Å, respectively). The steric clash among Leu7 and Tyr183 and Tyr212 may hamper the key interaction between the N-terminal segment and the activation loop of ZmCK2 α -4. Moreover, some other important interactions are absent in ZmCK2 α -4. In particular, instead of Arg5, Tyr7 and Tyr21 present in ZmCK2 α -1, there are Cys8, Trp10 and Thr24 in ZmCK2 α -4, disrupting the interactions with the backbone carbonyl group of Gln305 Asp140 and Glu175, respectively. Probably all these unfavorable changes in the N-terminal segment are responsible for the lack of constitutive catalytic activity of ZmCK2 α -4.

Effect of ZmCK2 α -4 on endogenous protein phosphorylation in maize extract

To determine the influence of ZmCK2 α -4 on phosphorylation of endogenous CK2 substrates, a maize protein extract (65 μ g) was pretreated with 50 μ M staurosporine (a potent wide spectrum protein kinase inhibitor whose efficacy on CK2 is unusually low) (Meggio et al. 1995), and the incubated in a kinase reaction buffer (without casein) as described in Materials and methods, in the absence or presence of GST:ZmCK2 α -4. As shown in Fig. 8, at least four proteins were phosphorylated by endogenous protein kinases insensitive to staurosporine, most probably by CK2. However, when GST:ZmCK2 α -4 was added to the protein extract, phosphorylation of three of those proteins (MW about 76, 48 and 40 kDa) was decreased, suggesting that ZmCK2 α -4 may function as a specific negative regulator of other active CK2s present in the extract. Interestingly, the labeling intensity of a 96 kDa protein was may be slightly increased.

Expression analysis of ZmCK2 α -4 and ZmCK2 α -1

Expression of ZmCK2 α -4 and ZmCK2 α -1 was studied by RT-PCR. The level of ZmCK2 α -4 and ZmCK2 α -1 mRNA was measured in samples of total RNA obtained from seedlings (4-day old), roots (4-day and 8-week old), and from leaves (2-week and 8-week old) of maize plants. ZmCK2 α -4 and ZmCK2 α -1 had different expression patterns. The ZmCK2 α -4 transcript was detected only in 8-week old leaves and roots, whereas ZmCK2 α -1 was found in all examined organs (Fig. 9).

Discussion

The present work reports the cloning of a transcript encoding a putative novel catalytic α subunit of CK2 from maize. The cDNA designated ZmCK2 α -4 was identified, purified and sequenced.

ZmCK2 α -4 was a full-length clone with an open reading frame beginning at three potential translation start codons and ending at a common stop codon. *ZmCK2 α -4* exhibited very high sequence homology to the previously found three catalytic subunits of maize CK2: *ZmCK2 α -1*, *ZmCK2 α -2*, and *ZmCK2 α -3*. A detailed analysis of all known sequences of maize CK2 α has revealed that *ZmCK2 α -4* and *ZmCK2 α -1* are produced by AS from the single gene *ZmPKCK2AL*. Both transcripts are composed of ten exons of which only the first one is different between them. The first exon of *ZmCK2 α -4* is located closer to the 5' end of *ZmPKCK2AL* than the first exon of *ZmCK2 α -1*. In plants, AS is an important regulatory mechanism controlling gene expression and eventually plant development and phenotype (Stamm et al. 2005; Reddy et al. 2007). Unlike promoter activity, which primarily regulates the amount of transcripts, alternative splicing changes the structure of the transcripts and their encoded proteins. One particular type of AS is the use of alternative first exons (AFEs) to regulate their expression and contribute to protein diversity. Studies concerning AFEs have mainly been focused on mammalian genomes, but recent studies show that AFEs also exist in plants. Based on large scale EST/cDNA alignments to the genomes of rice and *Arabidopsis*, it was estimated that at least about 5% of the expressed genes use AFEs (Chen et al. 2007). In the human, exon 1 of *CK2 α* gene is untranslated and the signal for translation start is located in exon 2. Two transcription start sites for *CK2 α* gene were identified upstream of the translation start site (position 1 and 50) (Wirkner et al. 1998). The presence of more than one transcription start sites is a common feature of TATA-less promoters (present in the human *CK2 α*), which usually contain a CG island and GC boxes (Wirkner et al. 1998). An untranslated first exon is also present in the *CK2 β* genes of human (Voss et al. 1991), *C. elegans* (Hu and Rubin 1991), and mouse (Boldyreff and Issinger 1995). A detailed analysis of the *Arabidopsis* genome has shown that some alternative splicing variants of *CK2 β 1* and *CK2 β 4* do exist (Salinas et al. 2006). This suggests that AS may also function for plant CK2 and expression of different variants of CK2 by that molecular mechanism is possible.

Our efforts were concentrated on evaluation whether *ZmCK2 α -4* exhibits a protein kinase activity. Therefore, variants I, II, and III of *ZmCK2 α -4* were expressed as GST fusion proteins in *E. coli* and purified on glutathione-Sepharose resin. Although high expression of all variants of *ZmCK2 α -4* was obtained, a major fraction of the recombinant proteins was found in an insoluble form in inclusion bodies. The soluble proteins were purified; however, the amount of pure *ZmCK2 α -4* proteins obtained was very low. Problems with solubility of CK2 expressed in *E. coli* as recombinant proteins were also observed in the case of *CK2 α* and *CK2 β* from *Drosophila* (Lin and Traugh 1993) and *CKA1* and *CKB1* from *Arabidopsis* (Klimczak et al. 1995). In both cases the proteins were solubilized with 8 M urea and renatured by diluting in an appropriate renaturation buffer. After solubilization, renaturation and purification these proteins were enzymatically active. The three variants of *ZmCK2 α -4* were also solubilized in 8 M urea, renatured by diluting in an appropriate renaturation buffer and purified, but the obtained proteins were not active (data not shown). Taking into consideration the fact that the

treatment with urea could influence the enzymatic activity of the proteins, for evaluation of the enzymatic activity of each variant (I, II, III) of GST:ZmCK2 α -4 only the fractions of soluble proteins were used. Neither of the tested variants of GST:ZmCK2 α -4 displayed an enzymatic activity with casein or specific peptide RRRDDDSDDD (data not shown) as substrates, even in the presence of human CK2 β in equimolar amounts with respect to the α subunit. That lack of activity could be caused by the GST tag at the N-terminus of ZmCK2 α -4, because any alterations in the N-terminal fragment cause a significant drop both in the catalytic activity and in thermal stability of CK2 α (Sarno et al. 2001). GST could prevent the interaction of the N-terminal part of ZmCK2 α -4 with the activation loop, and as a consequence a lack of enzymatic activity would be observed. Also, GST itself may disrupt the interaction between GST:ZmCK2 α -4 and CK2 β . Earlier results showed that ZmCK2 α -1 behaves like human CK2 α , forming a stable and active complex with human CK2 β (Boldyreff et al. 1993; Riera et al. 2003). To rule out the possibility that GST was responsible for the lack of enzymatic activity of ZmCK2 α -4, the intein IMPACT[®] system was used for the expression of ZmCK2 α -4 without a tag. Like the three variants of the GST:ZmCK2 α -4 fusion proteins, ZmCK2 α -4 devoid of a tag did not exhibit any enzymatic activity, which means that the presence of GST was not responsible for the lack of ZmCK2 α -4 enzymatic activity. Moreover, the fusion protein GST:ZmCK2 α -1 was active (data not shown). The reason of the observed lack of activity probably lies in the unusual N-terminus of ZmCK2 α -4 encoded by the first exon, because it is the only difference between ZmCK2 α -4 and ZmCK2 α -1. It has been reported that a tight contact of the N-terminus of CK2 α with the activation loop ensures open conformation and constitutive activity of CK2 α (Sarno et al. 2001). The presently elaborated model of ZmCK2 α -4 based on the crystal structure of ZmCK2 α -1 (Niefind et al. 1998) has shown that several important interactions present in ZmCK2 α -1 are lost in ZmCK2 α -4. Instead of Ala4, Arg5, Tyr7, and Tyr21 present in the ZmCK2 α -1 there are Leu7, Cys8, Trp10, and Thr24 in ZmCK2 α -4. According to our molecular models, these changes make the N-terminus of ZmCK2 α -4 unable to interact with the activation loop and to maintain it in an open and full active conformation. These observations are in agreement with the results of Sarno et al. (2002) showing that mutants of CK2 α with deletions in the N-terminal segment (Δ 2-18 and Δ 2-24) have almost completely lost the catalytic activity. Moreover, the activity of mutant Δ 2-18 can be totally restored upon association with the β -subunit, but mutant Δ 2-24 still displays a reduced catalytic activity. In the case of ZmCK2 α -4 all the favorable interactions between the N-terminal part and the activation loop reported for ZmCK2 α -1 and human CK2 α are absent. ZmCK2 α -4 shows a behavior similar that of the human mutant Δ 2-24, being inactive alone, in the presence of an excess of human CK2 β it is able to phosphorylate casein. This suggests that ZmCK2 α -4, similarly to the Δ 2-24 mutant, is able to interact with the β subunit and thereby to undergo a partial recovery of the catalytic activity. These results are consistent with those previously reported by Sarno et al. (2001),

showing that constitutive activity is conferred on CK2 α and on the CK2 holoenzyme through different molecular mechanisms. The holoenzyme is active even in the absence of interactions between the N-terminal segment and the activation loop. Most probably, in the holoenzyme the activation loop adopts its proper conformation independently of interactions with the N-terminal segment. Therefore, the activation of ZmCK2 α -4 by CK2 β makes possible that like ZmCK2 α -1, ZmCK2 α -4 may function not only as a monomer (a negative CK2 regulator), but also as a holoenzyme which catalytic activity is stimulated by specific regulatory subunit(s). Apart of an excess of CK2 β , the enzymatic activity of ZmCK2 α -4 can be partially restored by increasing the ATP concentration (K_m for ATP =1068 μ M). This is in agreement with the report that a deletion of 11 residues from the the N-terminal part of human CK2 α caused a loss of catalytic activity accounted for a dramatic rise in K_m for ATP (from 10 to 206 μ M) (Sarno et al. 2001; 2002). The affinity of ZmCK2 α -4 for ATP is two orders of magnitude lower than that of human CK2 α -1 and ZmCK2 α -1, whose K_m for ATP is 10 μ M and 13 μ M, respectively (Niefind et al. 1999; Sarno et al. 2002).

In an attempt to provide direct evidence for the existence of the ZmCK2 α -4 protein, specific antibodies were prepared. The 13-mer peptide YATVRRPGSYS DGS present in the N-terminal region of all three putative variants of ZmCK2 α -4 was used for rabbit immunization. The antibodies obtained specifically recognized recombinant GST:ZmCK2 α -4, but immunodetection of ZmCK2 α -4 in extracts of maize seedlings or leaves was not possible (data not shown). Most probably this was caused by too low amounts of ZmCK2 α -4 in the studied tissues. Thus, the presence of ZmCK2 α -4 protein in maize is still not documented. At the transcript level, both splice variants of the *ZmCK2 α* gene (*ZmPKCK2AL*), *ZmCK2 α -1* and *ZmCK2 α -4*, exist. In tobacco, two alternatively spliced transcripts of the *N* gene, a member of a class of resistance genes (R), are necessary to confer resistance to tobacco mosaic virus. In the resistance reaction, one transcript encoding the full-length protein was more prevalent after 3 h of virus infection, whereas the other splice variant encoding a truncated protein was more abundant 4 to 8 h after infection. Like in the case of ZmCK2 α -4, it is not known if these splice variants are translated (Dinesh-Kumar and Baker 2000).

Several reports concerning distribution of the CK2 α subunits in plants suggest their constitutive expression in all examined organs, but at different levels (Peracchia et al. 1999; Riera et al. 2001b; Salinas et al. 2006). The pattern of expression of *ZmCK2 α -1* and *ZmCK2 α -4* in various organs at different stages of development was studied by RT-PCR using specific primers. The obtained results indicate that while *ZmCK2 α -1* is present in every organ tested, *ZmCK2 α -4* is expressed differentially during maize development. The *ZmCK2 α -4* transcript was found in 8-week old maize leaves and roots, but not in 4-day old seedlings, roots, or 2-week old leaves. The fact that *ZmCK2 α -4* was preferentially expressed in older tissues explains its presence in the cDNA library made from 5-week

old leaves. Since CK2 is a positive regulator of proliferation, it is possible that in older organs when plant growth is slowed down, ZmCK2 α -1 is counteracted by the expression of ZmCK2 α -4.

We demonstrated that in maize ZmCK2 α -4 could function as a specific negative regulator of phosphorylation of some proteins by endogenous CK2s as well as displays some activity toward a small subset of proteins. We can speculate that phosphorylation of these proteins by ZmCK2 α -4 may play a role in limiting of proliferation. Considering that ZmCK2 α -4 could be a negative regulator of CK2 activity in plant cells at the protein level (as a competitor for CK2 substrates) or the RNA level (RNAi silencing of ZmCK2 α -1 expression), it is not surprising that the lowest level of ZmCK2 α -4 transcript is present in fast-growing and proliferating tissue (seedlings), and its expression is elevated in older leaves. To understand the exact roles of various splice variants of the *ZmCK2 α* gene in plant development, further studies are necessary.

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Legend to the figures

Fig. 1. Comparison of amino acid sequences of ZmCK2 α -4 with known CK2 α sequences from maize. The amino acid sequence of ZmCK2 α -1, ZmCK2 α -2, and ZmCK2 α -3 was aligned with ZmCK2 α -4. Identical residues are indicated by shaded boxes, and dash indicates a gap introduced to maximize alignment.

Fig. 2. CK2 α gene (*ZmPKCK2AL*) and two splicing variants of its mRNA.

(A) Exon I is absent in *ZmCK2 α -1*, whereas exon II is absent in *ZmCK2 α -4*. (B) The presented sequence corresponds to alternatively spliced 5'-terminal segments of *ZmCK2 α -4* and *ZmCK2 α -1* mRNAs. First exon of *ZmCK2 α -4* is located between nucleotides 1034 and 1137 in CK2 α gene. First exon of *ZmCK2 α -1* is located between nucleotides 1663 and 1747 in CK2 α gene.

Fig. 3. N-terminal sequences of putative variants I, II, III of ZmCK2 α -4.

Fig. 4. Expression and purification of ZmCK2 α -4, ZmCK2 α -1 and human CK2 β .

cDNAs of ZmCK2 α -4 (variants I, II, III), ZmCK2 α -1 and human CK2 β were over-expressed in *E.coli* and purified. Purification of GST:ZmCK2 α -4 (I-III) and ZmCK2 α -1 using S-hexylglutathione resin, ZmCK2 α -4 III using intein tag system, and human CK2 β on phosphocellulose column, was monitored by SDS-PAGE followed by Coomassie Brilliant Blue staining.

Fig. 5. Attempts at detection of enzymatic activity of ZmCK2 α -4.

(A) Analysis of kinase activity of GST:ZmCK2 α -4 (I-III) and ZmCK2 α -1 by in-gel kinase assay using casein as substrate was performed as described in Materials and methods. Upper panel shows autoradiogram and lower panel shows amount of recombinant proteins stained with Coomassie Brilliant Blue. Arrows indicate positions of recombinant proteins. (B) Phosphorylation of casein (in-solution assay) with GST:ZmCK2 α -4 (I-III), ZmCK2 α -4 (without tag), and ZmCK2 α -1 in absence or presence of human CK2 β in equimolar amounts with respect to α subunits. (C) Effect of an excess of human CK2 β on the activity of GST:ZmCK2 α -4 (variant III). The activity of ZmCK2 α -4 (0.1 μ g) was tested toward casein in the presence of β -subunit. CK2 β was added in equimolar amounts (lane 1), and in 3-fold (lane 2), 10-fold (lane 3) and 20-fold (lane 4) excess with respect to α -subunit. Phosphorylation conditions are described in Material and methods. Reaction products were subjected to 10% SDS-PAGE, visualized by Coomassie Brilliant Blue staining and analyzed by autoradiography.

Fig. 6. Determination of K_m of ZmCK2 α -4 for ATP.

Lineweaver-Burk plot is shown for various concentrations of ATP (250, 300, 500, 750, 1000 and 3000 μM) at the constant concentration of casein as substrate. The phosphorylation of casein was carried out as described in Materials and methods.

Fig. 7. Structural models of ZmCK2 α -1 and ZmCK2 α -4.

The most important interactions between the N-terminal segment (yellow) and the activation loop (violet) in ZmCK2 α -1 (A) are completely lost in ZmCK2 α -4 (B).

Fig. 8. Effect of ZmCK2 α -4 on phosphorylation of proteins in maize extract by endogenous CK2s.

Protein extracts from 2-week old maize leaves were pretreated (for 5 min) with 50 μM staurosporine or not (control), prior to incubation with [γ - ^{32}P]ATP and addition of GST:ZmCK2 α -4 (0.4 μg). Reaction products were separated electrophoretically on 10% SDS-PAGE, visualized by Coomassie Brilliant Blue staining and analyzed by autoradiography. CK2-phosphorylated proteins are identified by arrows.

Fig. 9. Expression of ZmCK2 α -4 and ZmCK2 α -1 in maize seedlings, roots and leaves.

Total RNA was extracted from seedlings (4-day old), roots (4-day and 8-week old), and leaves (2-week and 8-week old). The transcript level of ZmCK2 α -4 and ZmCK2 α -1 was determined by RT-PCR using specific primers and total RNA as template. For amplification of *actin*, primers from conserved regions of maize *actin* gene were used (Chang et al. 1999).

Figure 1

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CK2alpha-1 : -----MSKARVYADVNVLRPKBYWDYEALTVQWGEQDDYEVVRKVGRGKYSEVFEGINVNN : 56
CK2alpha-2 : -----MSKARVYTDVNVLRPKBYWDYEALTVQWGEQDDYEVVRKVGRGKYSEVFEGINVNN : 56
CK2alpha-3 : -----MSKARVYTDVNVLRPKBYWDYEALTVQWGEQDNYEVVRKVGRGKYSEVFEGINVNN : 56
CK2alpha-4 : MLAISVLCSWCDASGEVMTM--IYA--TVRRPGSYSD-----GSEQDDYEVVRKVGRGKYSEVFEGINVNN : 62

CK2alpha-1 : NEKCIIKILKPVKKKKIKREIKILQNLGGFNI VKLLD IVRDQHSKTPSLIFEYVNNNTDFKVL YPTLTDYDIR : 129
CK2alpha-2 : NEKCIIKILKPVKKKKIKREIKILQNLGGFNI VKLLD IVRDQHSKTPSLIFEYVNNNTDFKVL YPTLTDYDIR : 129
CK2alpha-3 : NEKCIIKILKPVKKKKIKREIKILQNLGGFNI VKLLD IVRDQHSKTPSLIFEYVNNNTDFKVL YPTLTDYDIR : 129
CK2alpha-4 : NEKCIIKILKPVKKKKIKREIKILQNLGGFNI VKLLD IVRDQHSKTPSLIFEYVNNNTDFKVL YPTLTDYDIR : 135

CK2alpha-1 : YYIYELKALDYCHSQGIMHRDVKPHNV MIDHELRLR LIDWGLAEFYHPGKEYNVRV ASRYFKGPELLVDLQ : 202
CK2alpha-2 : YYIYELKALDYCHSQGIMHRDVKPHNV MIDHELRLR LIDWGLAEFYHPGKEYNVRV ASRYFKGPELLVDLQ : 202
CK2alpha-3 : YYIYELKALDYCHSQGIMHRDVKPHNV MIDHELRLR LIDWGLAEFYHPGKEYNVRV ASRYFKGPELLVDLQ : 202
CK2alpha-4 : YYIYELKALDYCHSQGIMHRDVKPHNV MIDHELRLR LIDWGLAEFYHPGKEYNVRV ASRYFKGPELLVDLQ : 208

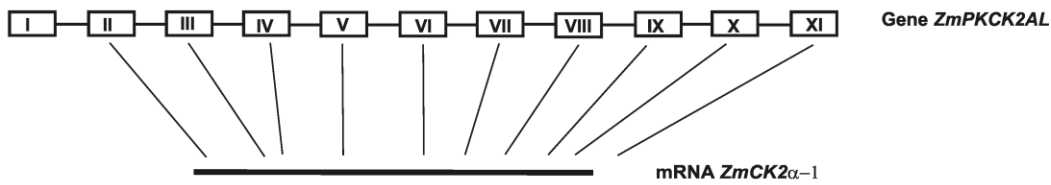
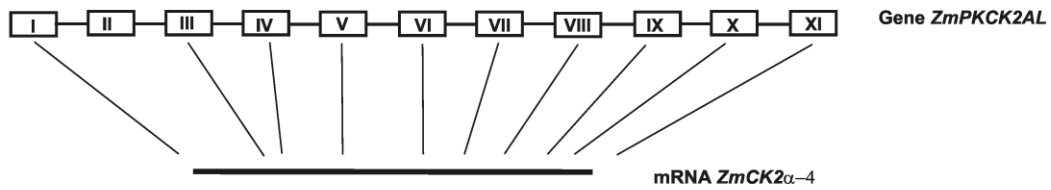
CK2alpha-1 : DYDYSLDMWSLGCMFAGMIFRKEPFFYGHDNHDQLVKIAKVLGTDGLN VYLNKYRIELDPQLEALVGRHSRKP : 275
CK2alpha-2 : DYDYSLDMWSLGCMFAGMIFRKEPFFYGHDNHDQLVKIAKVLGTDGLN VYLNKYRIELDPQLEALVGRHSRKP : 275
CK2alpha-3 : DYDYSLDMWSLGCMFAGMIFRKEPFFYGHDNHDQLVKIAKVLGTDGLN VYLNKYRIELDPQLEALVGRHSRKP : 275
CK2alpha-4 : DYDYSLDMWSLGCMFAGMIFRKEPFFYGHDNHDQLVKIAKVLGTDGLN VYLNKYRIELDPQLEALVGRHSRKP : 281

CK2alpha-1 : WSKFMNADNQHLVSP E AIDF LDKL LRYDHQ ERLTAL EAMTHPYEQQVRAAENSRTRA- : 332
CK2alpha-2 : WSKFMNADNQHLVSP E AIDF LDKL LRYDHQ DRLTAREAMHPYELQVRAAENSRTRPQ : 333
CK2alpha-3 : WSKFINADNQHLVSH E AIDF LDKL LRYDHQ DRLTAREAMTHPYEQQVRAAENSRTRA- : 332
CK2alpha-4 : WSKFMNADNQHLVSP E AIDF LDKL LRYDHQ ERLTAL EAMTHPYEQQVRAAENSRTRA- : 338

```

Figure 2

A



B

	1000	*	1020	*	1040	*
Y11649	Aaagcctcatcaggttcttctgctggaccacagctgagacctccatgcttgctataatctgtgctgtg					
AAF76187atgcttgctataatctgtgctgtg					
	1060	*	1080	*	1100	*
Y11649	cagctggtgtgacgcaagtggggaagtgatgacgatgatataatgctaccgtccggcggccggggtc					
AAF76187	cagctggtgtgacgcaagtggggaagtgatgacgatgatataatgctaccgtccggcggccggggtc					
	*	1140	*	1160	*	1180
Y11649	gtacagtgacgggaggtagaagataaagcacctatacctatccgtcaccacataatcttgtttgca					
AAF76187	gtacagtgacgggag.....					
	1640	*	1660	*	1680	*
Y11649	ctcgcgccggcaccgcatcggtcgggcccattgtccaaggccagggtctacgccgacgtcaacgtgct					
X61387atgtccaaggccagggtctacgccgacgtcaacgtgct					
	*	1720	*	1740	*	1760
Y11649	gcgccccaaggagtactgggattacgaggcgctcaccgttcaatgggggtggagtgctatctcgcgcgc					
X61387	gcgccccaaggagtactgggattacgaggcgctcaccgttcaatgggggtg.....					

Figure 3

ZmCK2α-4I	MIYATVRRPGSYSYDGS
ZmCK2α-4II	MTMIYATVRRPGSYSYDGS
ZmCK2α-4III	MLAISVLCWCDASGEVMTMIYATVRRPGSYSYDGS

Figure 4

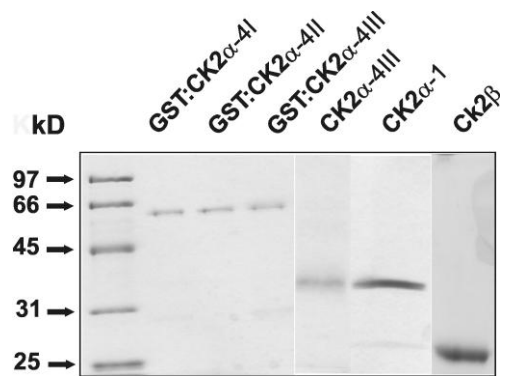


Figure 5

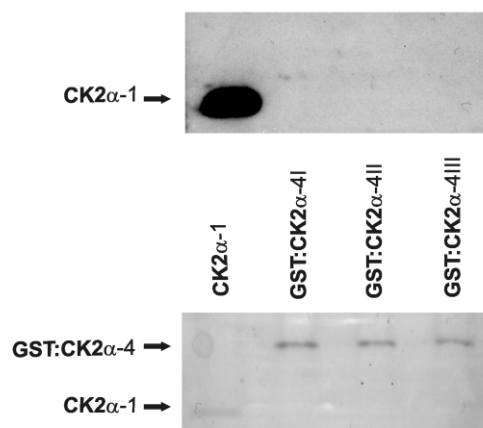
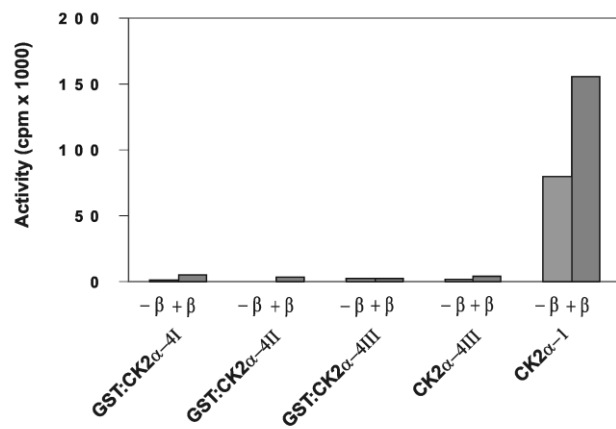
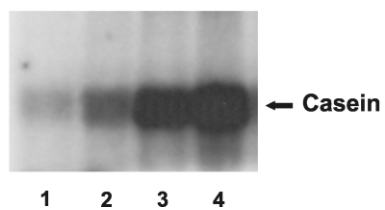
A**B****C**

Figure 6

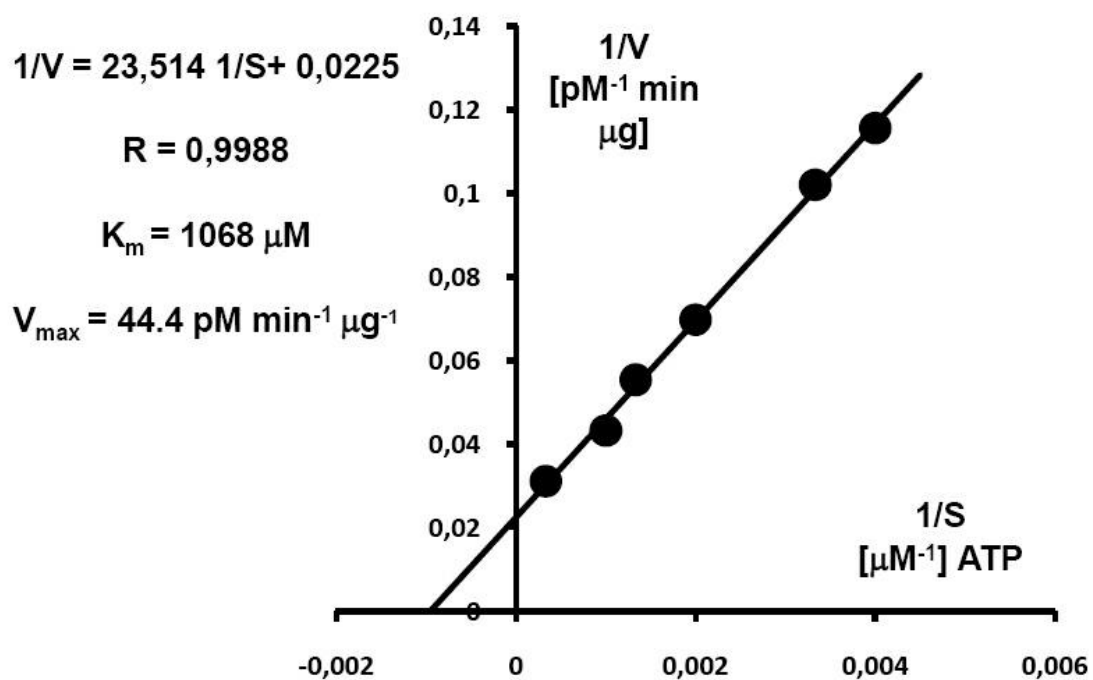


Figure 7

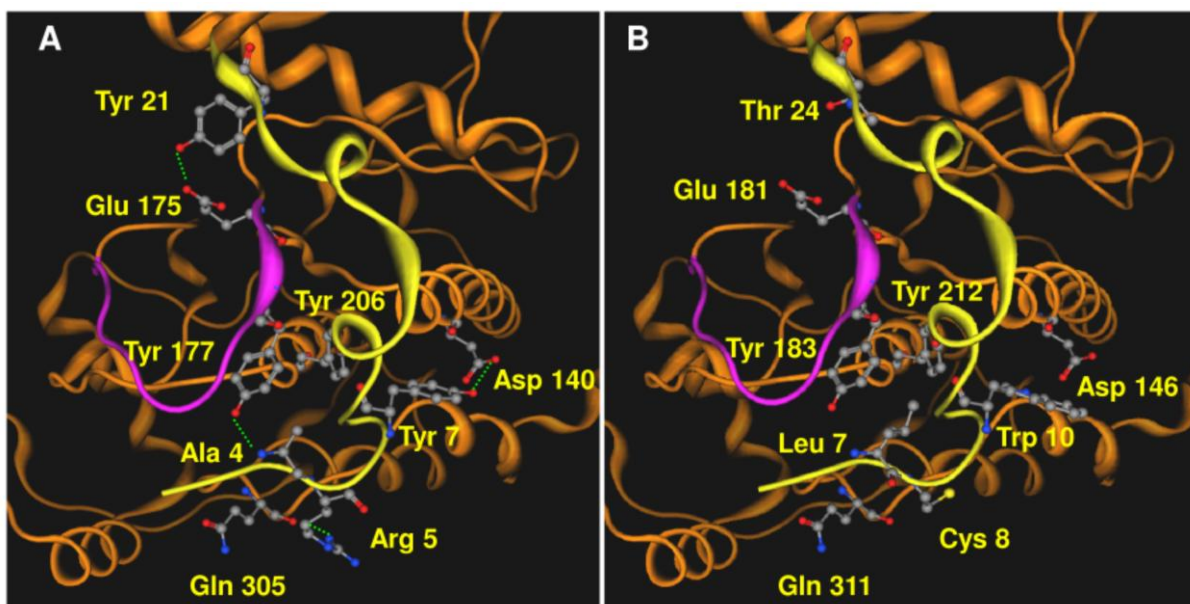


Figure 8

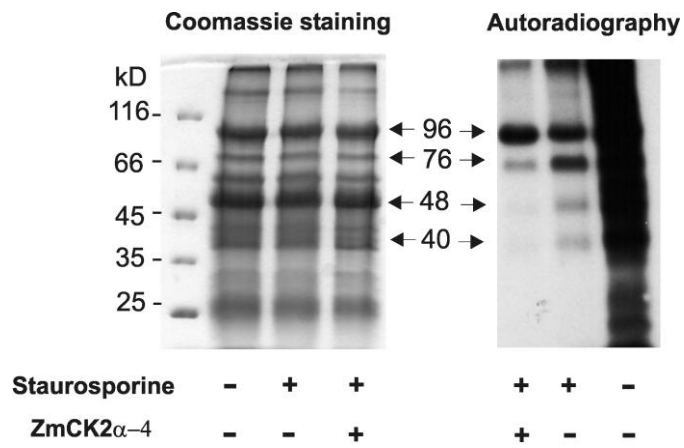


Figure 9

