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Purification and functional analysis of protein kinase G-1 α using a bacterial expression system

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Abstract

3',5' cyclic guanosine monophosphate (cGMP)-dependent protein kinase G-1 α (PKG-1 α) is an enzyme that is a target of several anti-hypertensive and erectile dysfunction drugs. Binding of cGMP to PKG-1 α produces a conformational change that leads to enzyme activation. Activated PKG-1 α performs important roles both in blood vessel vasodilation and in maintaining the smooth muscle cell in a differentiated contractile state. Recombinant PKG-1 α has been expressed and purified using Sf9-insect cells. However, attempts at obtaining full length protein in a soluble and active form using bacterial expression-purification systems have thus far been unsuccessful. These attempts were hampered by a lack of proper eukaryotic protein folding machinery in bacteria. In this study, we report the successful expression and purification of PKG-1 α using a genetically engineered *E. coli* strain, Rosetta gami 2(DE3), transduced with full length human PKG-1 α cDNA containing a C-terminal histidine tag. PKG-1 α expression was purified to homogeneity using sequential nickel affinity chromatography, gel filtration, and an ion exchange MonoQ. Western blot analysis and N-terminal sequencing revealed full length PKG-1 α after elution from the ion exchange column. Analysis of enzyme kinetics, using a nonlinear regression curve, identified that, at constant cGMP levels (10 μ M) and varying ATP concentrations, PKG-1 α had a maximal velocity (V_{max}) of 5.02 ± 0.25 pmol/min/ μ g and a Michaelis-Menten constant (K_m) of 11.78 ± 2.68 μ M ATP. Recent studies have suggested that endothelial function can be attenuated by oxidative and/or nitrosative stress but the role of PKG-1 α under these conditions is unclear. We found that PKG-1 α enzyme activity was attenuated by exposure to the NO donor, Spermine NONOate, hydrogen peroxide, and peroxynitrite but not by superoxide. The attenuation of PKG-1 α activity may be an under-appreciated mechanism in the development of endothelial dysfunction in cardiovascular disease.

Keywords

Cyclic GMP; Protein Kinase G; Rosetta gami; Purification

1. INTRODUCTION

3',5' cyclic guanosine monophosphate (cGMP) dependent protein kinase G (PKG) is a eukaryotic serine/threonine kinase involved in the regulation of diverse physiological functions, such as smooth muscle relaxation and platelet aggregation [1; 2]. PKG exists in

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¹PKG-1 α : Protein kinase G-1 α ; ONOO⁻: peroxynitrite; SpNONOate: spermine NONOate; XO: xanthine oxidase; SIN-1: 3-morpholino-sydnominine; GF: gel filtration; IPTG: isopropyl- β -D-thiogalactoside; trxB: theoredoxin reductase; gor: glutathione reductase

two forms derived from different genes: soluble PKG-I, a homodimer, and the membrane associated PKG-II, a monomer [2]. PKG-I has two isoforms: I α (75kD) and I β (78kD) which are products of alternate splicing [3]. The PKG-1 α variant is predominantly found in the lung, is more sensitive to activation by cGMP than PKG-1 β , and is the primary isoform involved in vasodilation [4; 5]. PKG has a number of functional domains. The C-terminal contains the catalytic domain consisting of the ATP and substrate binding sites. This domain catalyzes the transfer of γ -phosphate of ATP to serine/threonine residues in target proteins. The N-terminal regulatory domain of PKG contains two allosteric cGMP-binding sites, termed A and B. These sites are homologous to each other, and cGMP binding brings about a conformational change associated with kinase activation [6; 7; 8]. N-terminal to the regulatory domain is the dimerization motif and the auto-inhibitory site which contains a pseudo-substrate sequence that interacts with the catalytic domain to block access to substrate. This interaction keeps the kinase in an inactive state. cGMP binding to the kinase relieves the auto-inhibition and leads to its activation [6; 7; 8].

Although PKG-1 α has been successfully purified using a baculovirus-mediated expression system in Sf9-insect cells [9], attempts at purifying active enzyme using a bacterial expression-purification system have been unsuccessful [10]. These studies have shown that high expression of PKG-1 α can be achieved in *E. coli*, but most of the purified protein obtained was insoluble and catalytically inactive, even after modifying the solubilization and refolding conditions [10]. Thus, in contrast to eukaryotic cells, bacteria may lack the protein folding and/or post-translational modifications crucial for obtaining active PKG-1 α . In this study, we account the successful purification of full length human PKG-1 α in a soluble and active form using a modified derivative of the BL21 *E. coli* strain, Rosetta gami 2(DE3). These strains supply rare eukaryotic tRNAs for AGG, AGA, AUA, CUA, CCC, GGA codons to facilitate the expression of eukaryotic proteins and limit bacterial codon usage bias. These strains also have mutations in the thioredoxin reductase (trxB) and glutathione reductase (gor) genes to facilitate cytoplasmic disulfide bond formation and enhance protein folding that may be required to obtain activity in certain mammalian proteins.

2. MATERIALS AND METHODS

2.1 Construction of PKG-1 α cDNA

A cDNA of human PKG-1 α , corresponding to 671 amino acids, was synthesized by GENEART in the vector, pGA14. The C-terminal of the PKG-1 α gene was attached to a histidine tag to facilitate its purification. We flanked the cDNA with a *NheI/NotI* restriction sites for digestion and subsequent ligation into corresponding *NheI/NotI* sites in the kanamycin resistant pET28a vector. After sub-cloning from pGA14 to pET28a, a diagnostic digestion using *NheI/NotI* enzymes was used to confirm the presence of PKG-1 α cDNA. DNA sequencing was also performed to verify the correct sequence, and the plasmid was designated as pET28- PKG-1 α .

2.2 Expression and purification of PKG-1 α

Kanamycin resistant pET28-PKG-1 α was transformed into the *E. coli* strain, Rosetta gami 2(DE3) (Novagen). A single colony was picked and used to make a pre-inoculum in four sterile 50mL falcon tube containing 25mL of terrific broth (Sigma) and kanamycin (50 μ g/mL). Cultures were grown overnight at 37°C with shaking and were then used to inoculate an 8L culture of terrific broth also containing kanamycin (50 μ g/mL) in a fermenter. Cultures were grown at 30°C with aeration at 300rpm until an OD 600nm of 0.8 was reached. PKG-1 α expression was induced by the addition of isopropyl- β -D-thiogalactoside (IPTG, 0.75mM) and riboflavin (3 μ M). Cultures were then grown at 18°C for a further 8h in the dark. Cells were harvested by centrifugation at 1,400 $\times$ g at 4°C for 20min and resuspended

in lysis buffer containing 40mM Tris-HCl, 100mM NaCl, 5% glycerol, 1mg/ml lysozyme, bacterial protease inhibitor cocktail (Sigma), 32 units/mL DNase I (Sigma), 3 units/mL RNase A (Sigma), at pH 6.8. The bacterial cell suspension was incubated with mild shaking at 4°C for 30min. Cells were then disrupted by sonication using 3 × 1.5min pulses from a 40% amplitude microprobe (ultrasonic processor) followed by 3 cycles of freezing and thawing (30min at -80°C). Cell debris was removed by ultracentrifugation for 90min at 240,000 × g at 4°C. The supernatant was brought to 125ml with lysis buffer and loaded onto 2 × HisPrep™ FF 16/10 columns (GE Healthcare) with a 20ml bed volume preequilibrated with 5 × bed volume equilibration buffer (50mM EPPS, 100mM NaCl, 30mM imidazole, 5% glycerol, pH 6.8) in an AKTA FPLC Purifier System (Amersham Biosciences) equipped with a 150mL superloop (GE Healthcare) using a flow of 0.05mL/min. The column was then washed with approximately 5 × bed volume of washing buffer (50mM EPPS, 300mM NaCl, 30mM imidazole, 5% glycerol, pH 6.8) and was monitored by a UV detector until a baseline was reached. Bound protein was eluted with elution buffer (50mM EPPS, 300mM NaCl, 400mM imidazole, 5% glycerol, pH 6.8). The eluted protein was further purified utilizing a HiLoad™ 26/60 Superdex™ 200 prep grade gel filtration liquid chromatography column (GE healthcare) with a bed volume of 320ml. The mobile phase utilized was 50mM EPPS, 150mM NaCl, and 5% glycerol, pH 6.8 using a flow rate of 0.2ml/min. Sixty fractions of 5ml each were collected. SDS-PAGE in combination with Coomassie staining and Western blot analysis identified fractions 31-39 as containing PKG-1α. These fractions were pooled and concentrated using a Vivaspin-20ml concentrator (50,000 MWCO) (Sartorius Stedim Biotech). The concentrated protein was further separated using a Pharmacia MonoQ ion exchange column (10/100QL) pre-equilibrated with buffer A (20mM Tris-HCl, 1mM DTT, 5mM MgCl₂) using a flow rate of 0.5ml/min. Protein extract (1mg) was loaded on to the column using buffer A. The protein was eluted with a linear NaCl gradient of 0 to 1M in buffer A at a flow rate of 0.5ml/min. Forty fractions of 0.5ml each were collected. SDS-PAGE and Western blot analysis identified fractions 11-14 as containing PKG-1α. These fractions were pooled and concentrated using an Amicon Ultra-4 concentrator (ultracel 50,000 MWCO, Millipore), and 50μL aliquots were stored in 0.6mL microcentrifuge tubes (Axygen), snap frozen in liquid nitrogen, and stored at -80°C until further use.

2.3 Western Blot analysis

Protein samples were resolved using 4-20% Tris-SDS-Hepes polyacrylamide gel electrophoresis (PAGE), electrophoretically transferred to a Immuno-Blot™ PVDF membranes (Bio-Rad Laboratories), and then blocked with 5% nonfat dry milk in Tris-buffered saline (1h, room temp). The membranes were then subjected to Western blot analysis using a goat polyclonal anti-PKG-1α antibody (1:500 dilution, Santa Cruz). Reactive bands were visualized using chemiluminescence (Pierce Laboratories) on a Kodak 440CF image station. Band intensity was quantified using Kodak 1D image processing software.

2.4 N-terminal amino acid sequencing

Purified PKG-1α protein was subjected to SDS-PAGE and electrophoretically transferred to a PVDF membrane similar to Western blot analysis. The membrane was rinsed 5x with water, stained with Ponceau S, and the band corresponding to PKG-1α was excised for N-terminal microsequencing using Edman degradation with 494 Procise Protein Sequencer/140C Analyzer (Applied Biosystems Inc) at the Iowa State University protein facility.

2.5 PKG catalytic activity assay

The catalytic activity of the purified protein was determined using a non-radioactive immunoassay (Cyclex-MBL International Corporation), according to the manufacturer's

protocol. Briefly, protein samples were diluted in kinase reaction buffer containing Mg^{2+} and ATP (125 μ M) in the presence or absence of cGMP (10 μ M) and incubated in a 96 well plate pre-coated with a PKG substrate containing threonine residues phosphorylated by PKG. After incubation for 30min at 30°C to allow the phosphorylation of the bound substrate, an HRP conjugated anti-phosphothreonine specific antibody was added to convert a chromogenic substrate to a colorimetric substrate that was read spectrophotometrically at 450nm. The change in absorbance reflected the relative activity of PKG in the sample. The results were reported as pmols of phosphate incorporated into the GST-G substrate fusion protein by active PKG in the sample in the presence or absence of cGMP (10 μ M) per minute at 30°C per μ g of protein (pmol/min/ μ g). These results were extrapolated by comparing the spectrophotometrical values of the samples to the known activity (pmol/min) of a positive control. PKG activity was also determined in the presence of different reactive oxygen and nitrogen species. Briefly, purified protein was exposed to the superoxide generator, xanthine oxidase (XO, 100nM), the nitric oxide donor, Spermine NONOate (SpNONOate, 100 μ M), hydrogen peroxide (H_2O_2 , 100 μ M), or authentic peroxynitrite ($ONOO^-$, 1mM) for 10min on ice. The percent change in kinase activity compared to the untreated protein was then determined, as mentioned above.

2.6 Kinetic characterization of PKG-1 α

Kinetic constants were determined using nonlinear regression (curve fit) analysis (GraphPad Prism Software Inc). To determine the Michaelis-Menten constant (K_m) for ATP, the kinase assay was performed, as mentioned earlier; however the ATP concentration was titrated from 0-100 μ M, while keeping the cGMP concentration constant at 10 μ M.

3. RESULTS

A full length human PKG-1 α cDNA was subcloned into a pET28a vector and was used to transform the *E. coli* strain, Rosetta gami 2(DE3). To determine the most favorable conditions for protein induction, different concentrations of IPTG were added to the bacterial culture and incubated overnight at 18°C. Immunoblot analysis demonstrated that maximum protein expression was obtained using 0.75mM IPTG (Fig. 1A). This dose was used to further establish the optimum duration of IPTG induction. Our data show that 8h of IPTG (0.75mM) treatment at 18°C resulted in optimal expression of PKG-1 α (Fig. 1B). Under these conditions, PKG-1 α was then expressed in Rosetta gami 2(DE3) cells and purified to homogeneity by passing the bacterial lysate through a sequential purification scheme consisting of affinity chromatography, gel filtration, and ion exchange. Coomassie staining demonstrated an approximate 75Kd band in the ion exchange column elute (Fig. 1C). Anti-PKG-1 α antibody detected the band at 75Kd upon immunoblot analysis (Fig. 1D). The elution of the full length PKG-1 α was also confirmed by N-terminal sequencing of the first 5 amino acids using Edman degradation. An increase in the basal kinase activity (-cGMP) was identified after each purification step (Fig. 2). However, a cGMP dependent increase in catalytic activity was only observed from the MonoQ elute (Fig. 2). These results suggest that most of the kinetic activity in the crude, nickel, and gel filtration elutes was due to proteolytic fragments of PKG-1 α , which may lack the N-terminal and hence cGMP dependence. The increase in enzyme activity corresponded with a significant increase in the protein purification factor after each step (Table 1). At constant cGMP levels (10 μ M) and varying ATP concentrations, an analysis of enzyme kinetics using a nonlinear regression curve demonstrated that the purified PKG-1 α had a maximal velocity (V_{max}) of 5.02 ± 0.25 pmol/min/ μ g and a Michaelis-Menten constant (K_m) of 11.78 ± 2.68 μ M ATP (Fig. 3). Finally, we found that PKG-1 α kinase activity was attenuated upon treatment with the NO donor, SpNONOate (100 μ M), H_2O_2 (100 μ M), and $ONOO^-$ (1mM) but not with the superoxide donor, XO (100nM) (Fig. 4).

4. DISCUSSION

The data presented in this study demonstrate that full length human cGMP-dependent PKG-1 α can be expressed and purified in a soluble and catalytically active form using a bacterial expression-purification system. We found that 0.75mM IPTG induction for 8h at 18°C resulted in the optimal expression of PKG-1 α in the modified *E. coli* strain, Rosetta gami 2(DE3). The identity of the purified protein was established by four criteria: (a) upon SDS-PAGE the purified protein migrated to an apparent molecular weight of 75 kD; (b) the purified protein was detected by an antibody against PKG-1 α ; (c) N-terminal protein sequencing performed by Edman degradation identified the sequence corresponding to human PKG-1 α ; and (d) the kinase activity increased 5 times upon incubation with cGMP, suggesting that the purified protein was enzymatically active and subject to cGMP mediated regulation.

PKG-1 α is an important mediator in the NO/cGMP pathway and plays a major role in smooth muscle relaxation [2] and synaptic plasticity [11]. The enzyme has been cloned, expressed, and purified from eukaryotic cell lines [12] but attempts at its purification from prokaryotes have been unsuccessful [10]. Most of the purified protein was reported to be in an insoluble and physiological inactive form [10]. Efforts at solubilization and refolding by directing the export of protein into the periplasmic space or controlling the expression of the heterologous protein using a tac promoter did not yield a soluble or active protein [10]. Several factors in prokaryotic cells can impair the correct folding and purification of eukaryotic PKG-1 α protein. Firstly, codon usage by bacteria can limit the translation of human PKG-1 α mRNA. Secondly, *in vitro* studies have demonstrated that PKG is a labile protein susceptible to proteolytic digestion [13]. Therefore, the highly concentrated proteases of the bacterial intracellular environment may affect the expression and activity of full length PKG-1 α in *E. coli*. Thirdly, prokaryotes may lack systems for post-translational modifications, such as disulfide bond formation and/or phosphorylation that are crucial for productive protein folding and/or activation. For example, comparative studies in cAMP dependent protein kinase A (PKA) have shown that the two regulatory 1 (R1) subunits are covalently cross-linked with two disulfide bonds under oxidizing conditions [14]. The formation of these disulfide bonds between cysteine residues induced the subcellular translocation and activation of PKA independent of cAMP [15; 16]. Similarly, it has been proposed that cysteine 43 of PKG forms an interchain-, while cysteines 117, 195, 312, and 518 form intrachain-, disulfide bond(s) that render the kinase catalytically active independent of cGMP [17; 18; 19]. In our present study, we used the genetically engineered strain of *E. coli*, Rosetta gami 2(DE3). This *E. coli* strain supplies an increased copy number of limiting tRNAs for 7 rare codons (AGA, AGG, AUA, CUA, GGA, CCC, and CGG); thereby, alleviating codon usage bias by prokaryotes. Lack of intracellular Lon protease and outer membrane OmpT protease reduces the degradation of heterologous proteins expressed in this strain. Finally, Rosetta gami have mutations in both the thioredoxin reductase (*trx*B) and the glutathione reductase (*gor*) genes, which greatly enhance disulfide bond formation in the *E. coli* cytoplasm by maintaining an oxidized environment. The combination of these properties enhances protein expression and proper protein folding in the prokaryotic cytoplasm. However, although this strain is devoid of outer membrane OmpT and ATP-dependent Lon proteases, our data in combination with other studies [13] demonstrate that a significant amount of purified PKG-1 α is proteolytically cleaved. These findings suggest that other cytoplasmic proteases may be rendering the degradation of the protein, thereby lowering the yield of full length PKG-1 α . Further studies are warranted to optimize the expression and improve the purification of PKG-1 α in prokaryotes.

Previous studies have further demonstrated that PKG is particularly susceptible to proteolytic digestion at the N-terminal [13]. Dimeric PKG is rapidly cleaved by trypsin,

resulting in the formation of degradation fragments of 67kD, 55kD, and a dimeric N-terminal fragment of 18kD [13]. In the PKG-1 α isoform, trypsin preferentially cleaves at arginine 77 of the hinge region [20]. The resulting monomeric fragment (67kD) retains similar kinase activity as the full length PKG-1 α [21]. Although truncated PKG-1 α can still bind to cGMP, it is independent of cGMP mediated regulation, and is therefore constitutively active [21]. This fragment is also reported to lack the cooperative nature of cGMP binding [21]. In our present study, upon SDS-PAGE and Coomassie staining of crude, nickel, and gel filtration elutes, we identified abundant fragments of 67kD and 55kD in addition to the full length PKG-1 α . Interestingly, in these elutes, we also found that the PKG catalytic activity did not increase upon incubation with cGMP, suggesting that the majority of the PKG-1 α protein in these elutes consisted of truncated PKG-1 α fragments that lacked cGMP mediated activation. However, the ion exchange column elute resulted in the purification of full length PKG-1 α , corresponding to 75kD, which was activated upon the addition of cGMP.

The role of oxidative stress in undermining endothelial function is well known [22; 23]. In our previous studies, we have shown that the NO donor, SpNONOate, and the ONOO⁻ donor, 3-morpholino-sydnnonimine (SIN-1), mimic the nitration mediated decrease in PKG activity seen in lambs during inhaled NO therapy and in lambs with pulmonary hypertension secondary to congenital heart disease [22]. In our present study, we found that the catalytic activity of our purified PKG-1 α also decreased upon treatment with SpNONOate and ONOO⁻. In addition, our results demonstrated that PKG-1 α activity did not change upon treatment with the superoxide donor, XO. However, incubation with H₂O₂ significantly decreased the enzyme activity. These results corroborate a previous study where it was shown that the oxidation of PKG-1 α by H₂O₂ increased the affinity of the enzyme for substrate, but the phosphotransferase activity of the kinase was reduced in the presence of cGMP [17]. Thus, under conditions of oxidative and nitrosative stress occurring in various cardiovascular diseases, PKG inhibition may occur, and this may be a relatively understudied mechanism by which endothelial dysfunction occurs.

In conclusion, we have developed a novel protocol for the expression and purification of full length human PKG-1 α using the *E. coli* strain, Rosetta gami 2(DE3). This protein could be useful in future studies to determine the mechanism by which reactive oxygen and nitrogen species attenuate PKG activity.

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Fig. 1. Purification of recombinant PKG-1α from the *E. coli* strain, Rosetta gami 2(DE3)
 PKG-1α expression was induced with different doses of IPTG (0.1-1mM) at 18°C. After an overnight incubation, cells were harvested, lysed, and the protein resolved using SDS-PAGE and electrophoretically transferred to a PVDF membrane. The membrane was then probed with an anti-PKG-1α antibody. IPTG (0.75mM) induction resulted in maximum protein expression (A). The time-course of PKG-1α expression was then determined using 0.75mM IPTG (0-16h, 18°C). Immunoblot analysis demonstrated that maximum protein expression was achieved after 8h of induction (B). PKG-1α protein was then purified using these conditions by passing the crude extract through a series of purification columns including an affinity, a size exclusion gel filtration (GF), and an ion exchange MonoQ, as described in *Methods*. Elutes from each purification step were resolved by SDS-PAGE. Coomassie staining indicated the elution of a single band, corresponding to 75kD from the ion exchange column (C). Western blot analysis identified this 75kD band as PKG-1α (D). The antibody also identified other bands at 55kD and 43kD, which are likely proteolytic fragments of PKG-1α (D).

Fig. 2. Cyclic GMP dependent protein kinase activity of purified PKG-1α

PKG-1α kinase activity in the crude bacterial extract, affinity chromatography elute, size exclusion gel filtration (GF) elute, and the ion exchange MonoQ elute was measured by an ELISA, as described in *Methods*, in the presence or absence of cGMP (10μM). Basal catalytic activity (-cGMP) increased after each purification step from crude extract to MonoQ. However, cGMP dependent kinase activity (+cGMP) increased only in the protein eluted from the ion exchange column. Data are mean ± SEM, n=3.

Fig. 3. Kinetic characterization of purified recombinant human PKG-1α

The enzyme kinetics of PKG-1α from the ion exchange column was determined by titrating the ATP concentration from 0-100μM, while maintaining the cGMP levels constant (10μM). The change in the enzyme activity with each increasing dose of ATP was plotted using nonlinear regression (curve fit) analysis. The phosphotransferase reaction had a maximum velocity (V_{max}) of 5.02 ± 0.25 pmol/min/μg and a Michaelis-Menten constant (K_m) of 11.78 ± 2.68 μM ATP. Data are mean $\pm$ SEM, n=3.

Fig. 4. Effect of reactive oxygen and nitrogen species on the catalytic activity of purified recombinant human PKG-1α

PKG-1α was pre-incubated with XO (100nM), SpNONOate (100μM), H₂O₂ (100μM), or ONOO⁻ (1mM) on ice for 10min, and then the kinase activity was measured. Exposure of PKG-1α to the NO donor spermine NONOate (SpNONOate), hydrogen peroxide (H₂O₂), and peroxynitrite (ONOO⁻) attenuated enzyme activity, while no change was seen in enzyme activity upon treatment with xanthine oxidase (XO). Data are mean ± SEM, n=3. *P<0.05 vs. untreated.

Table 1
Summary of the purification scheme of recombinant human PKG-1 α purified from the *E. coli* strain, Rosetta gami 2(DE3)

Crude extract was obtained from 8L of bacterial culture. Total and specific activity was measured in the presence or absence of cGMP (10 μ M). By using the total activity (+cGMP) in the crude extract as 100%, we found that the yield dropped to 19.45% in the nickel, 3.36% in the gel filtration (GF), and 2.26% in the MonoQ elute. However, the purification factor increased 2.55 fold in the nickel, 5.95 fold in the GF, and 55.83 fold in the MonoQ elute from the crude extract (+cGMP).

Step	Protein (mg)	Total activity (pmol/min)		Specific activity (pmol/min/mg)		Yield (%)	Purification factor
		-cGMP	+cGMP	-cGMP	+cGMP		
Crude	855	83625	82812	98	97	100	-
Nickel	65.17	14902	16106	229	247	19.45	2.55
GF	4.83	2487	2785	515	576	3.36	5.95
MonoQ	0.34	427	1869	1234	5407	2.26	55.83