ISOLATION, OVEREXPRESSION AND CHARACTERIZATION OF AN ALKALINE-STABLE LIPASE KV1 FROM *Acinetobacter haemolyticus*

KALAIVANI BATUMALAIE

UNIVERSITI TEKNOLOGI MALAYSIA

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KALAIVANI BATUMALAIE

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Specially dedicated to:

My lovely family for their endless support and motivation.

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ABSTRACT

The study reports the purification and comprehensive biochemical characterization of a novel lipase KV1 (LipKV1) from *Acinetobacter haemolyticus* strain KV1. Strain KV1 was identified as *Acinetobacter haemolyticus* based on results of 16S rDNA sequencing, phylogenetic and BIOLOG. The intracellular wild-type LipKV1 was purified to offer specific activity of 32 U/mg with an estimated relative molecular mass of 37 kDa. The PCR product of LipKV1 revealed that the retrieved sequence contained the proposed complete lipase gene sequence at nucleic acid positions 1~954. The purified wild-type LipKV1 exhibited a maximum relative activity at 40°C and pH 8.0. The lipase was activated (112-128%) in Na^{+}, Ca^{2+}, K^{+} and Mg^{2+} and the enzyme hydrolyzed a wide range of oils with tributyrin (140%) being the preferred ones. Reducing (PMSF, DTT, β-mercaptoethanol) and chelating (EDTA) agents significantly inhibited the LipKV1 relative activity ($p < 0.05$). Surfactants Tween 20-80 (110-143%) significantly enhanced the relative activity ($p < 0.05$). Gene encoding intracellular lipase was cloned to produce a large quantity of the recombinant LipKV1. The lipase which contained His-tag was expressed in *Esherichia coli* BL21 (DE3) cells using pET-30a as expression vector. Using the central composite design, screening and optimization of induction conditions (cell density before induction, IPTG concentration, post-induction temperature and post-induction time) were performed. All parameters were significant ($p < 0.05$) in influencing the expression of LipKV1, rendering a 70% increase in enzyme production at optimum induction conditions. The expressed recombinant LipKV1 was purified using Ni-affinity chromatography, to a specific activity of 233.4 U/mg and an estimated relative molecular mass of 39 kDa. The recombinant LipKV1 exhibited a maximum activity at 40°C and pH 8.0. Homology modeling of the lipase structure was carried out based on the template structure of a carboxylesterase from the archaeon *Archaeoglobus fulgidus*, which shares a 58% sequence identity to LipKV1. The LipKV1 model comprised a single compact domain consisting of seven parallel and one anti-parallel β-strand surrounded by nine α-helices. Three conserved active-site residues, namely Ser165, Asp259, and His289, and a tunnel through which substrates access the binding site were identified. Docking of the substrates tributyrin and palmitic acid into the active site of LipKV1 modeled at pH 8.0 revealed an aromatic platform responsible for the substrate recognition and preference towards tributyrin. The binding modes from the docking simulation appear to correlate well with the experimentally determined hydrolysis pattern, for which pH 8.0 is optimum and tributyrin being the preferred substrate. A low K_m value (0.6 mM) for tributyrin further verifies the high affinity of LipKV1 for the substrate. Biophysical characterization of recombinant LipKV1 protein using ultaviolet-visible (UV-Vis) spectroscopy, circular dichroism (CD), fluorescence spectroscopy, ANS fluorescence spectroscopy, Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) indicated that the lipase retains its secondary structure and good folding at alkaline pH conditions (pH 8.0 and pH 12.0) and at 40°C. Alkaline-stable enzymes such as LipKV1 are therefore, useful in biotechnology-based industries in order to shorten production time, minimizing energy consumption and preventing undesired chemical transformations.

ABSTRAK

Kajian ini melaporkan penulenan dan pencirian biokimia yang komprehensif lipase KV1 baharu (LipKV1) daripada *Acinetobacter haemolyticus* strain KV1. Strain KV1 telah dikenalpasti sebagai *Acinetobacter haemolyticus* berdasarkan keputusan penjujukan 16S rDNA, filogenetik dan BIOLOG. LipKV1 jenis liar intrasel telah ditulenkan untuk memberikan aktiviti spesifik 32 U/mg dengan jisim molekul relatif anggaran 37 kDa. Produk PCR LipKV1 mendedahkan bahawa jujukan yang diperoleh mengandungi jujukan gen lipase lengkap yang dicadangkan pada kedudukan asid nukleik 1~954. LipKV1 jenis liar yang ditulenkan menunjukkan aktiviti relatif maksimum pada suhu 40°C dan pH 8.0. Lipase itu telah diaktifkan (112-128%) di dalam Na^{+}, Ca^{2+}, K^{+} dan Mg^{2+} dan enzim ini menghidrolisiskan pelbagai jenis minyak dengan tributirin (140%) sebagai pilihan. Agen penurunan (PMSF, DTT, β-merkaptoetanol) dan agen pengkelat (EDTA) telah menghalang dengan ketara aktiviti relatif LipKV1 ($p < 0.05$). Surfaktan Tween 20-80 (110-143%) telah meningkatkan aktiviti relatif dengan ketara ($p < 0.05$). Kod gen lipase intrasel telah diklon untuk menghasilkan sejumlah LipKV1 rekombinan. Lipase yang mengandungi tag-His telah dieksperasikan dalam sel *Esherichia coli* BL21 (DE3) menggunakan pET-30a sebagai vektor ekspresi. Menggunakan reka bentuk komposit pusat, penyaringan dan pengoptimuman keadaan induksi (ketumpatan sel sebelum induksi, kepekatan IPTG, suhu pasca induksi dan masa pasca induksi) telah dilakukan. Semua parameter adalah signifikan ($p < 0.05$) dalam mempengaruhi ekspresi LipKV1, menghasilkan peningkatan sebanyak 70% dalam pengeluaran enzim pada keadaan induksi optimum. LipKV1 rekombinan tereksperasi telah ditulenkan menggunakan kromatografi Ni-afiniti, memberikan aktiviti spesifik sebanyak 233.4 U/mg dengan jisim molekul relatif anggaran 39 kDa. LipKV1 rekombinan menunjukkan aktiviti maksimum pada 40°C dan pH 8.0. Seterusnya, pemodelan homologi struktur lipase telah dilakukan berdasarkan templat struktur karboksilesterase dari arkaeon *Archaeoglobus fulgidus*, yang berkongsi identiti urutan sebanyak 58% dengan LipKV1. Model LipKV1 mempunyai satu domain padat tunggal yang terdiri daripada tujuh rantaian β selari dan satu anti-selari yang dikelilingi oleh sembilan α-heliks. Tiga residu tapak aktif yang terpelihara, iaitu Ser165, Asp259, dan His289, dan terowong yang mana substrat mengakses tapak aktif melaluinya telah dikenalpasti. Kemasukkan substrat tributirin dan asid palmitik ke dalam tapak aktif LipKV1 yang dimodelkan pada pH 8.0 menunjukkan suatu platform aromatik yang bertanggungjawab untuk pengenalpastian substrat dan pilihan terhadap tributirin. Mod pengikatan daripada simulasi kemasukan nampaknya terkait rapat dengan corak hidrolisis yang ditentukan secara eksperimen, yang mana pH 8.0 ialah yang optimum dan tributirin merupakan substrat pilihan. Nilai K_m yang rendah (0.6 mM) bagi tributirin mengesahkan lagi kepilihan tinggi LipKV1 terhadap substrat tersebut. Pencirian biofizik terhadap rekombinan LipKV1 menggunakan spektroskopi ultra ungu-cahaya nampak (UV-Vis), dikroisma pekeliling (CD), spektroskopi pendarfluor, spektroskopi pendarfluor ANS, spektroskopi inframerah transformasi Fourier (FTIR) dan kalorimetri pengimbasan perbezaan (DSC) menunjukkan bahawa lipase tersebut mengekalkan struktur sekundernya dan lipatan yang baik pada keadaan pH alkali (pH 8.0 dan pH 12.0) dan pada suhu 40°C. Oleh itu, enzim terstabil alkali misalnya LipKV1 adalah berguna dalam industri berasaskan bioteknologi untuk memendekkan masa pengeluaran, meminimumkan penggunaan tenaga dan mengelakkan transformasi kimia yang tidak diingini.

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LIST OF ABBREVIATIONS

°F	-	Fahrenheit
%	-	Percentage
˚C	-	Celsius
A_{nm}	-	Absorption spectroscopy at nm light source
ANOVA	-	Analysis of variance
ANS	-	8-Anilinonaphthalene-1-Sulfonic Acid
APS	-	Ammonium Persulfate
BLAST	-	Basic Local Alignment Search Tool
BLASTn	-	Basic Local Alignment Search Tool – Nucleotide
bp	-	Base pairs
BSA	-	Bovine serum albumin
BSA	-	Bovine Serum Albumin
Ca	-	Calcium
$CaCl_2$	-	Calcium chloride
$CaCl_2$	-	Calcium chloride
CCD	-	Central Composite Design
CCRD	-	Central Composite Rotatable Design
CD	-	Circular Dichroism
CTAB	-	Cetrimonium Bromide

DEAE-C	-	Diethylaminoethyl Cellulose
DNA	-	Deoxyribonucleic acid
dNTPs	-	deoxynucleotide triphosphates
DSC	-	Differential Scanning Calorimetry
DTT	-	Dithioreitol
E.q	-	Equation
EDTA	-	Ethylenediaminetetraacetic Acid
EtBr	-	Ethidium Bromide
FTIR	-	Fourier-Transform Infrared Spectroscopy
GMS	-	Glycerol Monostearate
h	-	Hour
HCl	-	Hydrochloride
His-tag	-	Histidine-tag
IPTG	-	Isopropyl β-D-1-Thiogalactopyranoside
K_2HPO_4	-	Dipotassium Phosphate
kDa	-	Kilodalton
kg	-	Kilogram
KH_2PO_4	-	Monopotassium Phosphate
LB	-	Luria broth
LipKV1	-	Lipase KV1
MD	-	Molecular Dynamic
MEGA6	-	Molecular Evolutionary Genetics Analysis Software
min	-	Minutes

mL - Milliliter

mL - Milliliter

mM - Millimolar

mM - Millimolar

NaCl - Sodium Chloride

NaOH - Sodium hydroxide

NCBI - National Center for Biotechnology Information

ng - Nanogram

Ni-NTA - Nickel Sepharose Affinity

nmol - nanomole

NMR - Nuclear Magnetic Resonance

PCR - Polymerase Chain Reaction

PCR - Polymerase Chain Reaction

PDB - Protein Data Bank

pKa - Acid Dissociation Constant

PMSF - Phenylmethylsulfonyl Fluoride

*p*NPP - *p*-nitrophenyl palmitate

R^2 - Coefficient of determination

R^2 - Coefficient of determination

rpm - Revolution Per Minute

rpm - Revolution Per Minute

RSM - Response Surface Methodology

RSM - Response Surface Methodology

s	-	Second
SDS	-	Sodium Dodecyl Sulfate
SDS- PAGE	-	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SLS	-	Sodium Laureth Sulfate
TEMED	-	Tetra Methyl Ethylene Diamine
T_m	-	Thermal Transition Midpoint
UV	-	Ultraviolet
UV-VIS	-	Ultraviolet -visible
v/v	-	Volume Percentage per 100mL volume
vol	-	Volume
w/v	-	Weight per 100mL Volume Percentage
xg	-	Times gravity
ΔC_p	-	Heat Capacity
µmole	-	Mikromole
µg	-	Microgram
µL	-	Microliter
$CaCl_2.2H_2O$	-	Calcium Chloride Dihydrate
$MgCl_2.6H_2O$	-	Magnesium Chloride Hexahydrate,
$MgSO_4.7H_2O$	-	Magnesium Sulfate Heptahydrate
$(NH4)_2\ SO_4$	-	Ammonium Sulfate
$(NH_4)_2SO_4$	-	Ammonium Sulfate
$(NH_4)_2SO_4$	-	Nitrogen Isotopes in Ammonium Sulfate
3D	-	Three-Dimensional

16S rDNA	-	16 subunit ribosomal deoxyribonucleic acid

DNA BASES :

A	-	Adenine
C	-	Cytosine
G	-	Guanine
T	-	Thymine
N	-	Any base; A or C or G or T
M	-	Amino; represented by either A or C
W	-	Pyrimidine; represented by either C or T
R	-	Purine; represented by either G or A

AMINO ACIDS:

A or Ala	-	Alanine
V or Val	-	Valine
L or Leu	-	Leucine
I or Ile	-	Isoleucine
F or Phe	-	Phenylalanine
W or Trp	-	Tryptophan
P or Pro	-	Proline
G or Gly	-	Glycine
S or Ser	-	Serine
T or Thr	-	Threonine

C or Cys	-	Cysteine
Y or Tyr	-	Tyrosine
N or Asn	-	Asparagine
Q or Gln	-	Glutamine
K or Lys	-	Lysine
R or Arg	-	Arginine
H or His	-	Histidine
D or Asp	-	Aspartic acid
E or Glu	-	Glutamic acid

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Enzymes are valuable catalysts produced by nature, having numerous importance in improving our daily lives due to their high specificity as well as the economic advantages with limited adverse environmental impact. To date, there are ~ 4000 known enzymes and of these, almost 200 are in commercial use. Among the commercial enzymes, ~75 % of all industrial enzymes are hydrolytic and most of are microbial origin (Gurung *et al.*, 2013). The rising demand for industrial enzymes has been largely associated with their versatility to catalyze a multitude of processes. This has been attributable to enzyme-mediated processes being considerably better substitutes over the tedious and more expensive chemical route. The increasing numbers of industrial establishments switching over to using enzymes to catalyze a variety of industrial processes indicates that such trend may likely to continue into the future (Mariod and Salaheldeen, 2017).

The demand for microbial origin industrial enzymes is rapidly gaining popularity owing to their superiority for applications in industries on commercial scales (Sangeetha *et al.*, 2011; Prasad and Roy, 2018). Microbial enzymes are the preferred choice of enzyme over those from other living organisms mainly due to a number of advantages *viz.* the enzymes exhibit a myriad of catalytic activities, high production capacity within a short time span and ease for genetic manipulation as compared to enzymes that originated from plants or animals. In addition, microbes producing enzymes grow rapidly on inexpensive growth media (Nigam, 2013).

Current market scenario for hydrolytic enzymes has positioned lipases at the top third rank after proteases and amylases, with annual estimated economic values reaching ~USD590.5 million by 2020 (Bhosale *et al*., 2016). Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) are key enzymes in biotechnology owing to their multifaceted properties which cover a wide array of industrial applications *viz.* food technology, detergent, chemical industry and biomedical sciences (Bornscheuer, 2018). Lipases catalyze hydrolysis of long chain triglycerides at the interface between the insoluble substrate and water, forming diacylglyceride, monoglyceride, glycerol and free fatty acids as products. They also catalyze the enantio- and regioselective hydrolytic reactions as well as synthesize a broad range of natural and non-natural esters (Gaur *et al*., 2016). Lipase-producing microorganisms identified so far, included bacteria, fungi and yeasts with lipases isolated from *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium* and *Pseudomonas* receiving greater attention in the body of literature (Casas *et al*., 2012).

Bacteria producing lipases typically are isolated from environments which are rich in lipids or triglycerides. The bacteria eventually develop the ability to break down these organic materials, which ability is later harnessed for the biotransformation of a variety of compounds into beneficial or value-added materials (Bancerz *et al*., 2016). In this regard, the study was focused on the isolation of lipase-producing bacterium for possible utilization in manufacturing processes. In this study, the lipase producing bacteria *Acinetobacter haemolyticus* was isolated from an oil palm mill effluent (43°C and pH 8.4), an environment naturally rich in triglycerides. This bacterium is coccobacilliary, aerobic and gram-negative bacterium belonging to the wider class of Gammaproteobacteria (Park *et al*., 2009; Anbu *et al*., 2011). The bacterial species is oxidase-negative, exhibiting twitching motility (Bancerz *et al*., 2016) and typically occurring in pairs. *Acinetobacter sp.* grows optimally at 33 – 50 °C with a pH range of pH 5 – 9. The species is widely distributed in nature and commonly isolated from various sources that include soil (Tabaraki *et al.,* 2013) and water (Li *et al*., 2014). For instance, oil contaminated soil samples (Margesin *et al*., 2003), polluted fresh water (Zhang *et al*., 2015), sea water (Yoon *et al*., 2007), raw milk (Dharmsthiti *et al*., 1998) and clinical samples (Hostacka, 2000). However, initial designations of the bacteria have been rather sporadic, where much of the designations were founded on at least

15 different names. These designations have been occasionally cited in medical literature, in which the most frequently used names are *Bacterium anitratum*, *Mima polymorpha, Herella* (or *Herellea*) *vaginicola*, *Achromobacter, Diplococcus* B5W, *Micrococcus calcoaceticus* and *Cytophaga*. Later a French group from the Pasteur Institute proposed a slightly clearer taxonomy based on the biological tests on morphology, nutrition, and in vitro growth characteristics; that was later confirmed by the Subcommittee on the taxonomy of Moraxella (Towner, 1992).

Lipases from *Acinetobacter sp.* have been found useful for the bioremediation of alkanes and aromatic hydrocarbons, but their applications in other fields such as in detergent formulation, food industry and organic synthesis remain limited (Snellman *et al.*, 2002; Saffarian *et al.*, 2015). Previously, *Acinetobacter sp.* lipases were used to synthesize powerful emulsifiers from high molecular weight heteropolysaccharides, while a recombinant *Acinetobacter* lipase, from the intestinal sample of *Cyprinus carpio*, was added as a component in aqua feed (Ran *et al.*, 2015). In general, many of the commercial applications of hydrolytic lipases have been as additives in detergents, food ingredients and flavor development for dairy products. Such applications require unique features of enzymes that include high substrate specificity as well as temperature and pH stability. In fact, lipases are often incorporated as components of detergent and dishwasher formulations for effective removal of fatty residues, useful in cleaning clogged drains as well as domestic usages (Bisht *et al.*, 2013).

Factually, fungal lipases have an optimum pH in the neutral or slightly acidic range, while bacterial lipases lean toward the neutral or slightly alkaline pH region. In the case of an alkaline-stable lipase, it is defined as a class of bacterial enzyme capable of surviving in alkaline (pH 8 – 11.5) for 1 h while retaining 60-90 %, of its activity, indicating the alkalo-stable nature of the enzyme. Unfortunately, it is quite rare to isolate bacterial capable of producing alkaline-stable lipases (Salameh and Wiegel, 2007). There is an increasing interest in lipases of Acinetobacter for the past decade following the growth of enzyme-related industries as well as the widening search for novel enzymes for specific applications (Saffarian *et al.*, 2015), although, studies limited to just isolation and empirical assessment to establish the biochemical properties of the lipase would no longer suffice. A more up to date approach may

include the use of computational protein modelling software for comprehending the association of structure-activity in the lipase protein. Consequently, the mechanism of lipase catalysis can be fully comprehended by analyzing, the three-dimensional (3D) structure of the lipase.

1.2 Problem Statement

Considering that a large number of industrial processes operates at relatively high alkaline pH conditions (Clark, 2001; Vadlamani *et al.*, 2017), as well as the limited studies and choices of alkaline-stable lipases to catalyze such reactions (Gupta *et al.*, 2004; Bancerz *et al.*, 2016), the quest for bio-prospecting and comprehensive characterization of novel bacterial lipases showing versatile biochemical properties, merits specific attention from both the scientific and industrial communities. Most importantly, despite the numerous studies on alkaline-stable lipases, reports on lipases from *Acinetobacter sp.* are scanty in comparison to the *Pseudomonas/Burkholderia* species.

Hence, this present study focused on *Acinetobacter haemolyticus*, isolated from an oil palm mill effluent that produced a lipase designated as lipase KV1 (LipKV1). A comprehensive molecular and structural level profiling on LipKV1 was carried out to establish and confirm its alkaline-stability; the first ever report detailing an alkaline-stable lipase from *Acinetobacter haemolyticus*. A matter of fact, previous works on lipase from such bacterium have shown that the genus predominantly produces lipases with a neutral pH being the optimum working condition (Snellman *et al.*, 2002; Park *et al.*, 2009; Anbu *et al.*, 2011). Compare to other commercial lipases, LipKV1 have extended alkaline stability as well as being a mesophile which moderate optimum temperature is very beneficial for wide range of industrial application. Hence, the isolation and characterization of this *Acinetobacter haemolyticus* and the alkaline-stable LipKV1 that it produces appear as an interesting study that would substantially contribute to enhancing the body of knowledge.

The comprehensive profiling (biochemical and biophysical properties) of the wild-type and recombinant LipKV1 from *Acinetobacter haemolyticus* reported in this study is pertinent for determining its suitability for catalyzing commercial reactions under industrial settings. Such information is valuable in elucidating the nature of adaptations occurring in the lipase as well as providing insights into the molecular organization and function of conserved lipase residues or protein architecture that imparted its alkaline-stable property.

1.3 Objectives of the Study

The objectives of this research were:

1. To isolate and characterize a wild-type alkaline-stable lipase producing bacterium.
2. To clone and optimize the overexpression of wild-type lipase gene.
3. To purify the wild-type and recombinant lipase, as well as characterize their biochemical and biophysical properties.
4. To carry out *in silico* assessment to predict and explain the three-dimensional structure of the lipase in relation to its alkaline-stability.

1.4 Scope of the Study

Bacterial strain KV1 was isolated from the effluent of an oil palm mill and perform biochemical analyses such as 16S rDNA, BIOLOG® and phylogeny to identify the bacterium strain KV1 is *Acinetobacter haemolyticus*. Next, confirmatory qualitative assessments using tributyrin, triolein and rhodamine B agar plates to identify the true lipase activity of LipKV1 were undertaken. Prior to characterization, the wild-type LipKV1 was purified using a consecutive two-step procedure by ammonium sulfate precipitation followed by DEAE-cellulose ion exchange chromatography, as protein sequence of LipKV1 was ascertained. Purified wild-type

LipKV1 was subjected to comprehensive physicochemical properties to determine its optimal catalytic conditions *viz.* optimal pH and temperature as well as the effects of substrates specificity, metal ions, surfactants and inhibitors, to carry out hydrolysis of oil-buffer emulsion.

LipKV1 gene was cloned into *Esherichia coli* BL21 (DE3) cells assisted by Response Surface Methodology (RSM) for factors of cell density before induction, IPTG concentration, post-induction temperature and post-induction time, using pET-30a as the expression vector to give the His-tag containing recombinant LipKV1. The overexpressed recombinant LipKV1 protein was then purified and biochemically characterized. Then proceeded to perform homology modeling of LipKV1 structure was based on the structure of a carboxylesterase from the archaeon *Archaeoglobus fulgidus* as the template. *In silico* docking followed by molecular dynamics stimulation were used to calculate the binding energies of the most and least preferred substrates in the active site of LipKV1 under different environmental pH values. Crucially, the LipKV1 model is important as evidence of its alkaline-stability evolutionary adaptation.

The present study also assessed the biophysical properties of LipKV1 using UV-visible (UV-VIS) spectroscopy, circular dichroism (CD), fluorescence spectroscopy, ANS fluorescence spectra, Fourier-transform IR spectroscopy (FTIR) and differential scanning calorimetry (DSC). Information was derived from the above mentioned analyses may indicate alterations in the protein folding and structure-function relationship of the lipase under varying environmental conditions. The results are useful for affirming results obtained earlier in the *in silico* investigation. The kinetic parameters of the purified recombinant LipKV1 provided insights into the molecular basis of the activity and stability of the recombinant LipKV1.

1.5 Significance of the Study

The information obtained from the *in silico* and biophysical evaluations on the recombinant LipKV1 from *Acinetobacter haemolyticus* strain KV1 reported here may prove relevant in understanding of the alkaline-stability of the lipase. It would improve the current understanding on the molecular adaptations that contributed to this unique ability in the bacterium of such genus. It is believed the alkaline-stable LipKV1 produced by *Acinetobacter haemolyticus* may have appealing characteristics for use in industrial applications. Being the pioneering work, our results would pave the way to the acceptance of LipKV1 in many forthcoming scientific as well as industrial endeavors.

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