Protein Expression and Purification 60 (2008) 117–126

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Recombinant proprotein convertase 4 (PC4) from *Leishmania tarentolae* expression system: Purification, biochemical study and inhibitor design

Ajoy Basak *, Nausheen J. Shervani, Majambu Mbikay, Maria Kolajova

Chronic Disease Program, Regional Protein Chemistry Centre, Ottawa Health Research Institute, U Ottawa, 725 Parkdale Avenue, Ottawa, Ont., Canada K1Y 4E9

ARTICLE INFO

Article history:
Received 5 December 2007
and in revised form 12 March 2008
Available online 25 March 2008

Keywords:
Proprotein convertase 4
Fertilization
Proteolytic activity
Fluorogenic substrates
Protease inhibitor
Proteomic analysis

ABSTRACT

Proprotein convertase 4 (PC4) is a member of Ca^{2+}-dependent mammalian subtilases called Proprotein convertases (PCs) or Proprotein convertases subtilisin kexin (PCSK). PC4 plays a key role in mammalian fertilization, sperm maturation and sperm–egg fusion. Full length and C-terminal truncated rPC4 have been expressed using *Leishmania tarentolae* expression system. Secreted soluble enzyme was recovered in good yield from concentrate medium and purified by DEAE anion exchange and arginine–agarose column chromatographies. This is the first attempt to produce rec (recombinant) PC4 by *Leishmania* expression system in reasonably pure and enzymatically active form. The eluted fraction contained PC4 protein as confirmed by immunoreactivity using PC4-specific antibodies. Two protein bands at ~62, 53 kDa in SDS–PAGE were attributed to C-terminal truncated PC4 forms. The fraction displayed strong protease activity towards fluorogenic Boc-RVRR-MCA and various intramolecularly quenched peptides derived from PC4-substrates. It also cleaved proIGF-2 to produce active IGF-2 confirming its role in this maturation process. Moreover PC4-mediated proteolysis was efficiently blocked by a newly designed prodomain $rPC4^{101-116}$ peptide with IC_{50} in low μM level. Similar but more potent PC4-inhibitory activity with K_i in low nM range was observed with the tetrapeptide chloromethyl ketones, Dec-RVKR/K-cmk (chloromethyl ketone). The study showed that such PC4 inhibitors may find potential therapeutic and clinical applications in male fertility.

Proprotein convertase 4 (PC4)[1] or Proprotein convertase subtilisin/kexin-4 (PCSK4) is a Ca^{2+}-dependent serine protease that cleaves inactive precursor proteins at the motif **R/K/H-X-X/R/K-R**↓, X = any amino acid except Cys [1] with **KXKR** being most preferred [2,3]. A schematic diagram showing various characteristic domains of PC4 is presented in Fig. 1. Among these, prodomain plays an important role as intramolecular chaperone and regulator of the catalytic activity of the enzyme. The removal of this domain is essential for the activity of the enzyme. The C-terminal P-domain plays an important role for proper folding and conformation of the molecule and subsequently controls the ultimate activity of the enzyme. PC4 activates protein precursors in reproductive secretory pathway [4,5]. Its restricted expression in testicular germ cells, ovarian macrophages and placenta suggests a possible reproductive function [4–8]. Its role in sperm maturation, sperm–egg interaction and fertilization has been well documented. PC4-KO (knock out) mice were subfertile with no apparent spermatogenic defect [9,10]. Sperms for PC4-KO mice have reduced ability to bind to zona pellucida, penetrate the cumulus mass and fertilize eggs. It is not clear whether PC4-activity is required for sperm–egg interaction, sperm production and fertilization although a number of physiological substrates in reproductive pathways have been associated with PC4. These include proPACAP [11], proIGF-1/2 [8,12] and ADAM (A Disintegrin And Metalloproteinase) proteins [13] ADAM-1 (fertilinα) [14], ADAM-2 (fertilinβ) [15], ADAM-3 (procyritestin) [16] and ADAM-5 [17]. For functional activity, these molecules require selected proteolysis. This is most likely mediated by PC4 and not by the sperm protease acrosin since mice lacking acrosin [18] are still fertile. So far only proPACAP [11] and proIGF-2 [8,12] have been confirmed as physiological substrates of PC4. Owing to these findings, the study of PC4 enzyme, its inhibitors and substrates has drawn considerable interest. Earlier we generated rec-PC4 in extremely poor yield using GH4C1 [2] and Hi5 cells [3]. We were only able to perform some initial *in vitro* work on the enzyme and biochemical activities, even though the enzyme was never purified. Herein we report the use of *Leishmania tarentolae* expression system (LEXSY) for improved PC4 production in active form [19]. This system allows (i) rapid gene amplification, (ii) posttranslational modifications, (iii)

* Corresponding author. Fax: +1 613 761 4355.
E-mail address: abasak@ohri.ca (A. Basak).

[1] *Abbreviations used:* AMC, 7-amino 4-methyl coumarin; CT, carboxy terminal; Dec, decanoyl; DEAE, diamino ethyl amino ethyl; FL, full length; h, human; KO, knock out; LEXSY, *Leishmania* expression system; MAP, multiple antigenic peptide; MCA, 4-methyl coumarin 7-amide; MW, molecular weight; m, mouse; NT, amino terminal; PC4, proprotein convertase 4; Pcsk4, proprotein convertase subtilisin/kexin 4; proIGF, proinsulin growth factor; proPACAP, propituitary adenylate cleavage activating peptide; r, rat; rec, recombinant; T, truncated.

1046-5928/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2008.03.013

stable expression in three convenient configurations, constitutive, inducible and secretory and finally (iv) non-pathogenic environment LEXSY has been used in the past to generate various recombinant proteins [19–21] but here for the first time using LEXSY, we produced a secreted soluble PC4 in enzymologically active form and purified it by chromatography for further biochemical characterization. We confirmed its identity by trypsin digestion followed by mass spectrometry [22], Western blot [2] and fluorescent in gel zymogram [23]. In addition, we also designed a potent PC4-inhibitor based on its prodomain sequence as we did for other PCs [24] and developed an additional polyclonal antibody for the enzyme.

Materials and methods

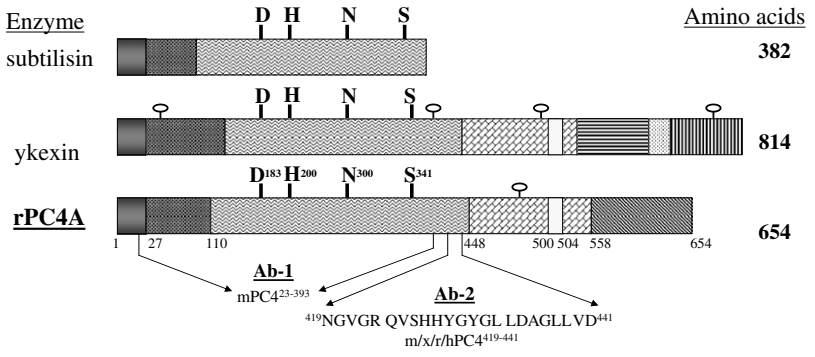
Materials

All amino acid derivatives (L-configuration), reagents, resin for peptide synthesis, peptidyl-MCA and cmk derivatives were purchased from Bachem Inc. (King of Prussia, PA, USA), Calbiochem Novabiochem Inc. (San Diego, CA, USA), Neosystems Inc. (CA, USA) or PE Biosystems (Foster City, CA, USA). IQF-peptides were prepared as described [3]. *L. tarentolae* gene expression kit was from Jena Bioscience GmbH, http://www.jenabioscience.com/cms/en/1/browse/1119_lexsy_literature.html, Germany. DEAE–sepharose and Arg–sepharose 6B were bought from Amersham Bioscience, Piscataway, NJ, USA. Anti-V5 and PC4 antibody, Ab-1 (against $mPC4^{23-393}$) were obtained from Alexis Biochemicals (San Diego, CA, USA) while the antibody Ab-2 (Fig. 1) was generated inhouse against $rPC4^{419-441}$ using MAP strategy [25]. Reagents for Western blot and SDS–PAGE were purchased from BIO-RAD Labs (Hercules, CA, USA). Matrix-Assisted Laser Desorption (MALDI) and Surface Enhanced Laser Desorption Ionization (SELDI) time-of-flight (tof) mass spectra (MS) plates were purchased from PE-Biosystems and Ciphergen, (Fremont, CA, USA), respectively. 1-Cyano 4-hydroxy cinnamic acid (CHCA) and 1,2-dihydroxy benzoic acid (DHB) were bought from Sigma Chemical Company, USA.

Expression and purification of rec-rPC4

Plasmids containing cDNA encoding full length (FL) and C-terminally truncated (T) rPC4 sequences (^{27}RPPI–CSAA^{654}-V5) and (^{27}RPPI–WDED^{546}-V5), respectively, with C-terminal V5-tag (designated as rPC4-FLV5 and rPC4-TV5) were amplified by transformation into *Escherichia Coli*. It was then stably transfected into *L. tarentolae* strain by electroporation. Recombinant strains harboring the constructs were cultured in brain heart infusion medium at concentration of 35 g/l, supplemented with 0.25% hemin in the presence of penicillin (10,000 units), streptomycin (10,000 μg/ml) and nourseothricin (100 μg/ml) at 26 °C in dark with shaking (90 rpm). Culture medium containing rec-PC4 was collected when cell confluence reached an OD_{600nm} value of 1.5–2.0. This constituted approximately 1×10^8 cells per ml of culture medium. The medium was centrifuged and the supernatant (~60 ml) was concentrated to 1.2 ml (~50-fold) by centricon (cut-off MW ~10 kDa) at 4 °C. Cell-bound PC4 was extracted with buffer containing 20 mM Tris–HCl (pH 7.5) + 150 mM NaCl + 1 mM Na_2EDTA + 1 mM EGTA + 1% Triton + 2.5 mM sodium pyrophosphate + 1 mM β-glycerophosphate + 1 mM Na_3VO_4 and 1 μg/ml leupeptin.

For purification, concentrate medium obtained from rPC4-FLV5 expression was adjusted to pH 8.0 and then loaded into DEAE–sepharose column (2.5 × 15 cm) pre-equilibrated with buffer 25 mM Tris + 25 mM Mes, pH 8.0 (buffer-A). Following washing with buffer-A (10× column volume), the column was treated successively with buffer-A + 25, 50 or 100 mM NaCl, respectively (30 × 1 ml each). Combined 100 mM salt pool (30 ml) containing most PC4-activity was concentrated to 4 ml by centricon and loaded into arginine–sepharose 6B column (2.5 × 15 cm), pre-equilibrated with buffer-A. Following extensive washing with buffer-A, the column was eluted with buffer-A containing arginine·HCl in increasing concentrations from 15 to 25 mM by a step of 2.5 mM (15 fractions 0.5 ml each). PC4 assay was performed by initial rate and/or end-time methods using 96-well plates (Dynatech, Millipore, USA) using fluorogenic Boc-RVRR-MCA [2] and IQF peptides [3].



Ab-1: PC4 polyclonal antibody commercial (Alexis Biochemicals) raised against its catalytic and middle-domain of $mPC4^{23-393}$ (99.5% homology with the corresponding rPC4 sequence).

Ab-2: Our PC4 polyclonal antibody developed against the species conserved region $m/x/r/hPC4^{419-441}$ located at far catalytic domain.

y = yeast, r = rat, m = mouse, h = human, x = xenopus laevis

Fig. 1. A schematic presentation showing the molecular architectures with various specific domains as indicated for rat (r) PC4 in comparison to the bacterial subtilisin and yeast kexin homologs. Ab-1, polyclonal antiPC4 antibody developed against the peptide segment (419–440) located at the P-domain of rat PC4 sequence; Ab-2, the commercial polyclonal antiPC4 antibody generated against the mouse sequence $PC4^{23-396}$ was obtained from Alexis Biochemicals Company.

SDS–PAGE, mass spectrometry and Western

PC4 samples were electrophoresed on 12% SDS–PAGE and stained with Coomassie dye as described [2,3]. In parallel, immunoblotting was performed after the bands were electro-transferred onto PVDF membrane within 120 min. Immunoreactivity between PC4 and antibody Ab-1 or Ab-2 was detected by horseradish peroxidase conjugated with mouse anti-human IgG [2,3]. For characterization purpose, Coomassie-stained-immunoreactive PC4 bands were excised and digested with trypsin as described (http://donatello.ucsf.edu/ingel.html) [22]. A gel band with no protein staining served as a control. The digests were subjected to SELDI-tof-MS and observed peaks were analyzed using peptide mass mapping.

Fluorescent in-gel zymogram

SDS–polyacrylamide gel containing peptide-MCA-substrate copolymerized in the gel were prepared as described in (23) with modification. Briefly, the resolving gel consisting of 200 μM Boc-RVRR-MCA, 10% acrylamide, 0.37% bis-acrylamide, 0.375 M Tris–HCl (pH 8.8) and 0.1% SDS was cast in mini-PROTEAN 3 cell—gel cassette (Bio-Rad Laboratories, Ltd., Mississauga, Ont., Canada). Polymerization was conducted in the dark for 1 h at room temperature. The 4% stacking gel contained 0.25 M Tris–HCl (pH 6.8), 0.03% SDS and no substrate. Purified rec-rPC4 (11.6 μg protein in 10 μl) was prepared in 1× sample buffer [0.25 M Tris–HCl (pH 6.8), 0.1% SDS and 2% glycerol] and was subjected to electrophoresis in 25 mM Tris–HCl (pH 8.3), 192 mM glycine and 0.1% SDS for 2 h at 20 mA constant current at 4 °C in the dark. The gel was then washed in cold 50 mM Tris (pH 7.4) and 2.5% Triton X-100 for 20 min, and in distilled water 3 × 5 min in the dark. The gel was incubated in assay buffer [50 mM Tris–HCl, 20 mM Hepes (pH 7.4), 10 mM $CaCl_2$ and 0.005% Triton X-100 at 37 °C for 30 min. Fluorescent band of rPC4-activity was observed by a Syngene (A division of Synoptic)-transilluminator (Cambridge, England) and fluorescent images were analyzed by Gene Snap version 6.01 software.

Peptide synthesis and antibody production

Peptides were synthesized by Fmoc-solid phase chemistry [2,3] using Fmoc-PAL-PEG resin for regular peptides and $Fmoc_4$-Lys_2-Lys-Ala-Wang resin for 4-branch MAP [25]. All IQF-peptides (listed below) were prepared according to [3]. (1) Q-hproIGF-1^{66-74}:Abz-PAKSAR↓SVR-Tyx-A, (2) Q-hproIGF-2^{63-71}:Abz-PAKSER↓DVS-Tyx-A, (3) Q-h/rPACAP$^{141-151}$:Abz-RVKNKGRR↓IAY-Tyx-A, (4) Q-hproPACAP$^{141-151}$-mut:Abz-RVKNKGRR↓IPS-Tyx-A, (5) Q-hproPACAP$^{99-111}$:Abz-DDSEPLSKR↓HSDG-Tyx-A, (6) Q-mproCyritestin$^{449-462}$:AbzTIAERGRLMR↓KSKD-Tyx-A, (7) Q-mFertilin α$^{428-440}$:Abz-EPGRQSRMRR↓AAN-Tyx-A, (8) Q-hFertilin β$^{438-450}$:Abz-FMSKERMMR↓PSFE-Tyx-A, (9) Q-mAdam-$5^{380-388}$:Abz-ERKPAR↓RPR-Tyx-A and (10) Q-mAdam-$5^{380-388}$-mut:Abz-E*P*KPAR↓RPR-Tyx-A (Abz, Ortho amino benzoic acid; Tyx, 3-nitro tyrosine; mut, mutant and Q, quench). For production of PC4 antibody Ab-2, 4-branch MAP-rPC4$^{419-441}$ (MW calculated 10,165, observed 10,184), as prepared above, was injected into rabbits as described in [25].

PC4 digestion and inhibition assay

Recombinant proIGF-2 and synthetic IQF peptides (10 μg each) were digested with purified rec-rPC4-FLV5 (2 μl, 25.65 U/mg), [1 U (unit) of activity is defined as one that releases 1 pmol AMC after 1 min digestion with 50 μM Boc-RVRR-MCA]. The reaction was terminated by adding acetic acid (2 μl) and kinetic parameters including K_i and IC_{50} values for PC4-inhibition were measured [2,3]. Each data point in the plots is the average of two independent experiments each performed in duplicate.

Results

Production of rec-rPC4

Presence of PC4 in *Leishmania* culture medium was detected by enzyme activity assay, protein content measurement, Western blot, SDS–PAGE analyses and finally by fluorescent in-gel zymogram.

Activity assay

As indicated by progress curves (Fig. 2A), rPC4-TV5 crude concentrate medium displayed strong protease activity towards fluorogenic substrates pERTKR-MCA, Boc-RVRR-MCA and RQRR-MCA (50 μM) as compared to control crude concentrate medium obtained from blank plasmid. Among these, pERTKR-MCA was cleaved with the highest degree of efficiency, being ~2.6- and 1.8-fold more potent than for the cleavages of RQRR-MCA and Boc-RVRR-MCA, respectively, under identical condition. Similar observations were also noted with stop-time assay (Fig. 2B) which also indicated a slightly lower level of activity (~1.7-fold less when measured against pERTKR-MCA) in rPC4-FLV5 crude concentrate medium compared to rPC4-TV5 crude medium (Fig. 2B). However in both cases a significant amount of PC4-activity remained attached to the cell membrane as demonstrated by its strong presence in cell lysate fraction. PC4-activity in either crude media was also detected with synthetic IQF-peptides derived from ADAMs and proIGF-2 processing sites (Fig. 2C–F) [3]. Among those tested, Q-fertilin-α and Q-ADAM-5 were both found to be the most potent for activity present in rPC4-TV5 crude media, while for rPC4-FLV5 crude medium Q-ADAM-5 alone was the best substrate. However Q-IGF-2 that contains "**KXXR**" motif [3] was found to be the most selective PC4-substrate, exhibiting no activity in control medium.

SDS–PAGE

Coomassie, stained gels of crude PC4-TV5 and PC4-FLV5 concentrate media exhibited bands at ~72 (for FL), 62, 53 and 38 kDa (Fig. 3A) largely consistent with the molecular sizes of various expected PC4 forms *(explained later)*. The results were further confirmed by immunoblots (Fig. 3B and C) using PC4-specific antibody. Calculated molecular weights of various potential forms of PC4 are indicated below. These included enzymatically inactive, active and C-terminal cleaved shed forms.

From full length rPC4 construct: rPC4$^{27-654}$+V5 (inactive full length) = 74,436 Da; rPC4$^{111-654}$ + V5 (active full length) = 64,214 - Da, rPC4$^{27-486}$ (inactive shed) = 51,534 Da and rPC4$^{111-486}$ (active shed) = 41,312 Da.

From truncated rPC4 construct: rPC4$^{27-546}$ + V5 (inactive truncated) = 59,852 Da and rPC4$^{111-546}$ + V5 (active truncated) = 49,630 and rPC4$^{111-486}$ (active shed) = 41,312 Da. In these calculations, RLIR^{486}↓SL was used as the potential PC4 shed processing site. This is based on its sequence alignment with furin and other PCs that are known to undergo shedding during their expressions.

Western blot

Immunoblotting on crude PC4 concentrate media and lysates is shown in Figs. 3B and C. V5 antibody failed to detect any immunoreactive PC4 band in the media suggesting the loss of V5-tag. However, strong anti-V5 immunoreactive bands were detected at ~72, 66 and 53 kDa in cell lysates indicating the presence of intact V5-tag PC4 proteins in this fraction. However with antibody Ab-1, immunoreac-

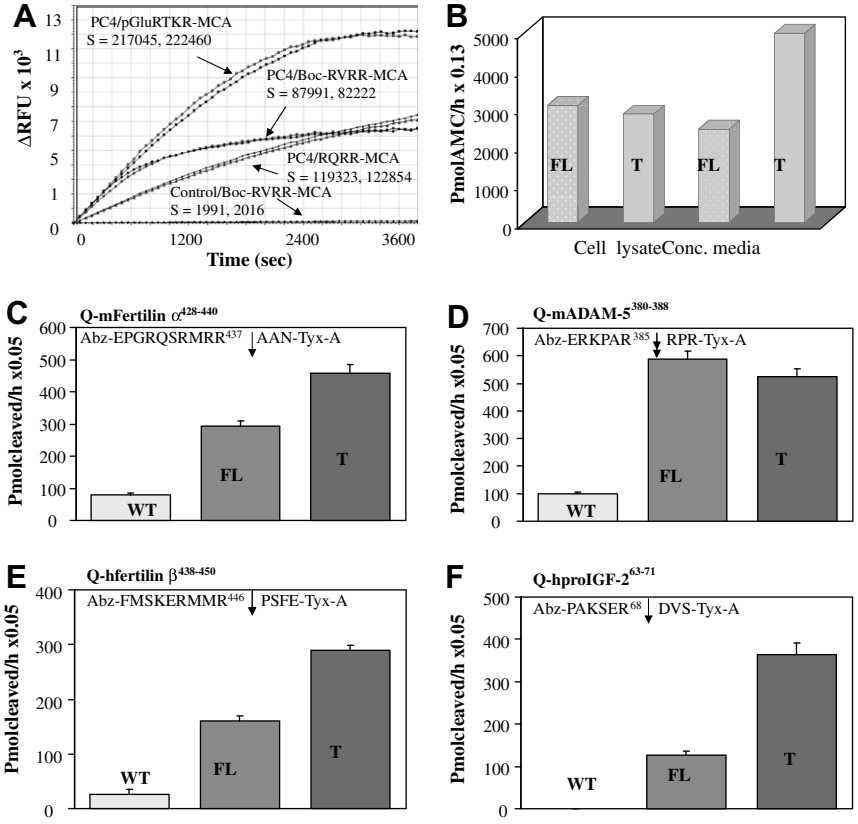


Fig. 2. (A) Progress curves showing online release of free AMC following incubation of crude rec-rPC4 with various peptidyl-MCA fluorogenic substrates (50 μM final concentration) over a 60 min reaction period. The measured slope (*S*) value of each obtained curve (done in duplicate experiments) representing the level of enzyme activity is shown in the figure. The enzyme activity corresponding to each curve was derived from 2 μl of crude concentrated (50-fold) *Leishmania* culture supernatant of truncated (T) rPC4-V5. The activity was monitored as described in [6]. (B) End time assay using Boc-RVRR-MCA (50 μM) (average of three independent experiments) for full length (FL) and truncated (T) rPC4 forms in the media and cell lysates. (C–F) Enzyme activity of 50-fold concentrated *Leishmania* rPC4-TV5 culture supernatant using different IQF-peptides derived from PC4 processing sites of ADAMs and other substrates. Each assay was performed using 2 μl of crude enzyme sample representing an activity of 50 U/μg protein [1 unit (U) of activity is defined as one that releases 1 pmol of free AMC following digestion with 50 μM Boc-RVRR-MCA for 1 min] and each IQF-peptide (50 μM concentration) in 25 mM Tris + 25 mM Mes buffer containing 2.5 mM $CaCl_2$, pH 7.4. ΔRFU = increase in raw fluorescent unit after 1 h of digestion, *S* = slope of progress curve.

tive bands at ~72, 62, 53 kDa were detected in truncated and full length rPC4 media as well as in corresponding cell lysates.

Kinetic study

Proteolytic activity of rec-PC4 in two crude concentrate media was assessed in more detail by measuring kinetic parameters for cleavage of Boc-RVRR-MCA. As determined by Michaelis–Menten kinetics [2,3], the measured V_{max} and K_m values (Fig. 3D and E) showed near identical kinetic efficiency for full length and truncated PC4 with $V_{max}/K_m = 7.2$ and $12.8 \times 10^{-9}\ h^{-1}$, respectively.

Purification of recombinant PC4

Although a significant amount of PC4 protein remained attached to the cell membrane as revealed by Western blot results on the cell lysate, attempts were only made to purify PC4 from the crude concentrate medium obtained from rPC4-FLV5. Although the cell lysate fraction contained a significant amount of PC4, we did not make any effort to purify it because of the complex nature of the fraction.

DEAE column

The anion exchange DEAE–sepharose was selected as the first column for purification of rec-PC4 obtained from the culture medium of rPC4-FLV5. Our choice was based on earlier studies showing decent results in purification of other recombinant PCs using this column [1–3]. Moreover like other PCs, PC4 possesses a high isoelectric point (for PC4-FLV5, calculated value = 8.36). After loading and extensive washing with buffer-A, bound PC4 was eluted from the column with buffer-A supplemented with 100 mM NaCl in fraction #(48–68) as revealed by activity and protein assays (Fig. 4A). This combined fraction was subjected to further purification by chromatography over arginine–sepharose 6B column that binds most of active PC4 protein. After washing of the unbound proteins, PC4 was released by incubation with buffer-A containing free arginine·HCl. Various elution conditions using stepwise increase in arginine concentration in buffer-A were pursued. However, most effective separation, as monitored by activity assay, was achieved with a 2.5-mM step gradient of arginine concentration beginning from 15 to 25 mM. Three separate peaks were eluted with 17.5–20 mM arginine·HCl containing buffer-A and fractions comprising these peaks namely #(106–115), #(116–124) and #(125–135) marked as E1, E2 and E3, respectively, were collected (Fig. 4B). These fractions contained mostly PC4 as revealed by SDS–PAGE with Coomassie protein staining and Western blot data (Fig. 5A and B). The data indicated the presence of three PC4-immunoreactive bands at ~62, 53 and 38 kDa in these fractions. Combined with corresponding Coomassie, stained SDS–gel electrophoresis, it is noted that the E1-pool contained a major band at ~62 kDa and two additional faint bands at ~52 kDa and 38 kDa due to the presence of PC4 proteins (Fig. 5A and B). As anticipated, these bands were completely absent in enzymatically inactive fractions #(20–30) and #(97–105) of arginine column. Measured en-

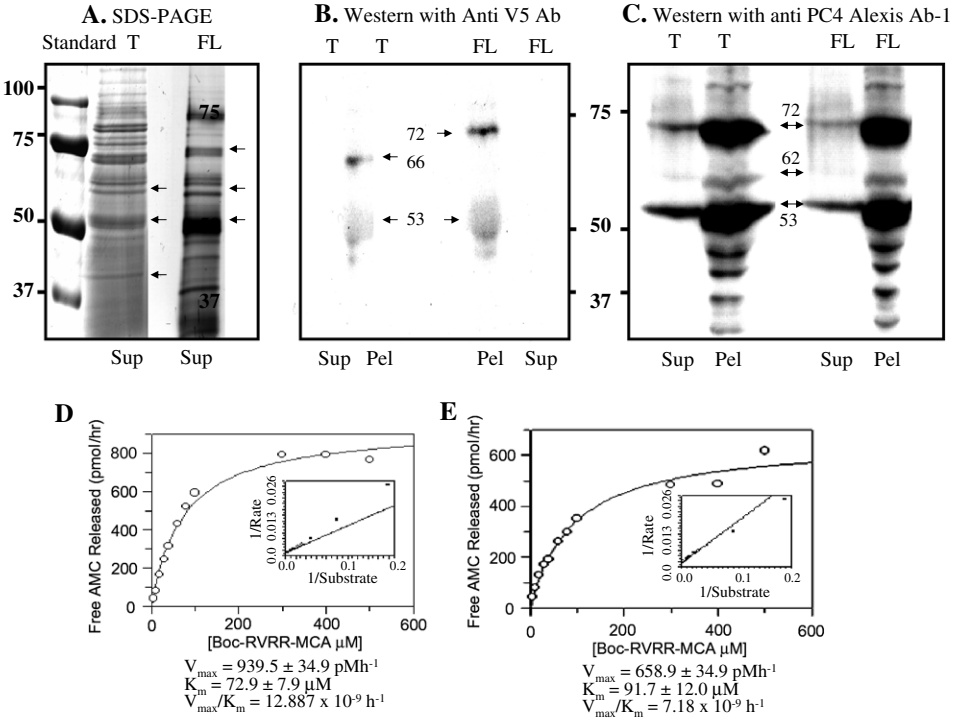


Fig. 3. (A) SDS–PAGE (12%) of crude concentrate media obtained from rPC4-FLV5 and rPC4-TV5 constructs following expression in *Leishmania* system. The bands are visualized by staining with Coomassie blue dye. PC4 protein bands were indicated by horizontal arrows. (B and C) Western blot analyses results of crude concentrated cell pellet extract and supernatant from *Leishmania* PC4 system using anti-V5 antibody and anti-human PC4 Ab-1 antibody. Sup, concentrated supernatant; Pel, cell pellet lysate; T, truncated PC4; FL, full length PC4. (D and E) Michealis–Menten graphs showing the kinetics of cleavage of Boc-RVRR-MCA at various concentrations by a fixed amount of crude PC4-TV5 and PC4-FLV5 concentrate media. The kinetic parameters V_{max} and K_m calculated from the graph are also displayed in the figure.

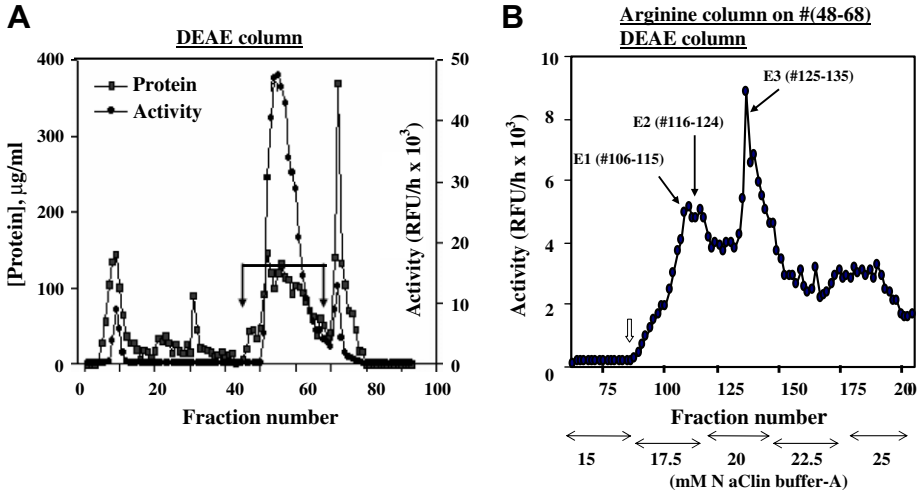


Fig. 4. (A) Chromatogram of *Leishmania* PC4-FLV5 crude concentrate medium through DEAE-sepharose column. The first arrow (↓) indicates the point where the column was treated with 100 mM NaCl in buffer-A for elution of PC4 protein following extensive wash (10 column volumes) with buffer alone (see Materials and methods). The solid square and circle symbols represent the protein and enzyme activity measurements, respectively, of various column fractions. Each fraction was of 0.5 ml size. (B) Chromatogram of combined 100 mM NaCl pool [fractions #(48–68)] of DEAE column containing PC4-activity through arginine–sepharose 4B column. The double vertical arrow indicates the start point where elution of PC4-activity began. The elution profile of PC4-activity under increasing concentrations of free arginine·HCl (as indicated in the figure) in buffer consisting of 25 mM Tris + 25 mM Mes + 2.5 mM $CaCl_2$, pH 7.4 to shown in the figure.

zyme and specific activities (Table 1) showed ~30–42-fold purifications of PC4 depending on the fractions (E1, E2 or E3) considered. Fluorescent in-gel zymogram assay with Boc-RVRR-MCA-substrate showed a positive band at ~62 kDa for fraction E1 suggesting that this band is proteolytically active that cleaves Boc-RVRR↓MCA at the site indicated and possibly belonged to a PC4-fragment derived from rPC4$^{110–678}$ + V5 (calculated molecular weight = 64,214) that has lost the V5-tag (Fig. 5C). Neither 53 kDa nor 38 kDa bands could be visible in the fluorescent zymogram suggesting that these are enzymatically inactive forms of PC4. While it is most likely that the 53 kDa may be the shed rPC4$^{27–486}$ (calculated MW = 51,534 Da), the identity of 38 kDa could not be ascertained at the present moment. Similar zymogram profile was also obtained for fractions E2 and E3 (not shown).

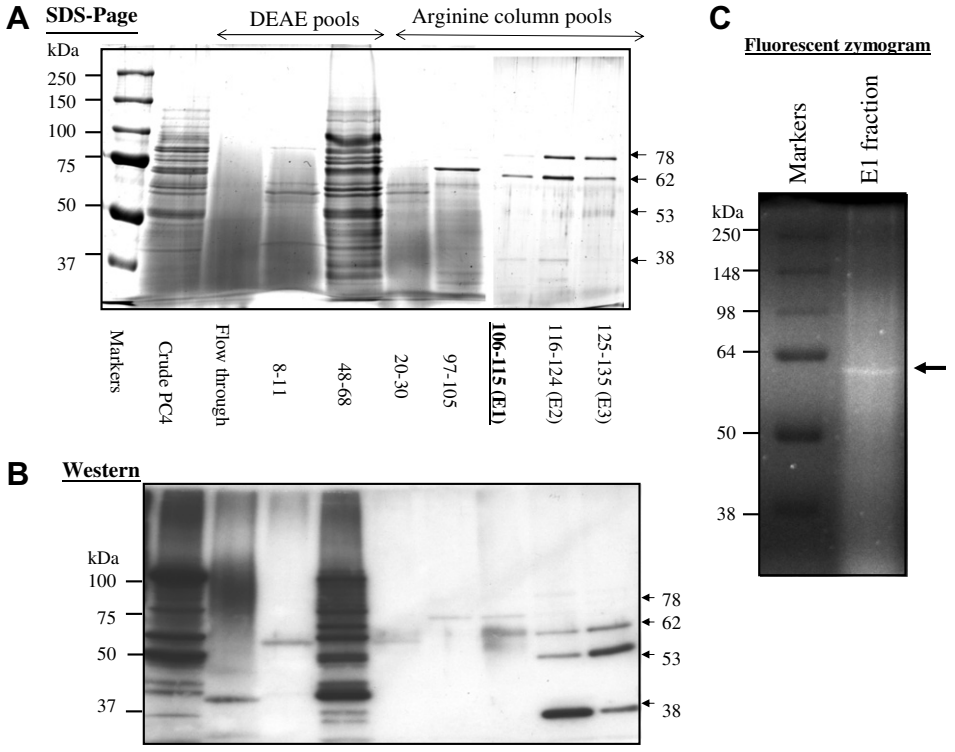


Fig. 5. (A) Coomassie-stained 8% SDS–PAGE of various fractions following chromatography of rec-PC4-FLV5 concentrate medium through DEAE and subsequently arginine–sepharose 6B columns as described in Fig. 4(B). (B) Western blot analyses of crude rec-PC4-FLV5 sample and various fractions including those containing PC4-activity obtained from DEAE and arginine–sepharose column chromatographies. The immunoblots were performed using commercial PC4 polyclonal primary antibody Ab-1 (Alexis Company). The immunoreactive PC4 bands at kDa ~72, 62, 53 and 38 in various fractions are shown by arrows. (C) Fluorescent in-gel zymogram assay of purified rec-rPC4 (E1 pool) using the fluorogenic substrate Boc-RVRR-MCA (200 μM) co-polymerized in 10% SDS–PAGE.

Characterization of PC4 bands

Identities of immunoreactive ~62 and 53 kDa bands as PC4 protein were confirmed by tryptic digestion followed by mass spectrometry results [22]. Thus the mass spectrum of crude digest of 53 kDa protein band exhibited peaks at m/z 1323, 1342, 1358, 1373, 1623, 1905, 2107, 2135, 3792 and 3908 (Supplementary Fig. 1A–C, not observed in the mass spectra of blank gel or trypsin auto-digests. These peaks were attributed to fragments $rPC4^{42-52}$, $rPC4^{148-160}$, $rPC4^{96-105}$, $rPC4^{111-122}$, $rPC4^{467-481}$, $rPC4^{487-502}$, $rPC4^{467-481}$, $rPC4^{42-73}$, $rPC4^{72-89}$ and $rPC4^{366-401}$, respectively (Supplementary Fig. 2), thus confirming that 53 kDa is a PC4 protein, most likely the inactive shed $rPC4^{27-486}$ (calculated molecular weight = 51,534 Da). Using a similar approach, 62 kDa protein band was identified as most likely the proteolytically active rPC4 protein that starts with Ser^{111} residue with the end amino acid at position 678 or an upstream position (calculated molecular weight for $rPC4^{111-654}$ = 62,812 Da). Above rationalization for 53 and 62 kDa bands was consistent with the fact that both lacked V5-tag as they were undetectable by V5 antibody and that only 62 kDa band was positive in the zymogram as expected. This observation suggested proteolytic truncation of full length rPC4 protein at an unknown site upstream of V5-tag by an enzyme endogenous to *Leishmania* system [26,27].

Processing by rec-rPC4-FLV5

proIGF2 processing: To test further the PC4-activity in E1-pool of Arg-column, it was incubated with rec-proIGF-2, a known physiological substrate of PC4 [8]. As expected, immunoblot data revealed that proIGF-2 (~14 kDa) was efficiently cleaved in a time-dependent manner yielding mature IGF-2 (7 kDa) (Fig. 6A). Presence of PC4-activity in E1-pool was further verified by studies using a PC4 inhibitor. As shown by progress curve assay, PC4 was inhibited in a dose-dependent manner by Dec-RVKR-cmk (a general PC-inhibitor), which at 5 μM concentration completely suppressed the activity present in 2 μl of E1-pool with activity 25.65 U/mg protein (Fig. 6B).

Processing of synthetic IQF peptides: All synthetic IQF peptides were efficiently cleaved by crude recPC4 and E1 fraction at the expected sites (see Materials and methods). As an example, this is shown in Fig. 7. for cleavage of Q-mFertilin-$\alpha^{428-440}$ by recPC4. MALDI-tof MS of PC4 digest of Q-mFertilin-$\alpha^{428-440}$ showed peaks at *m/z* 1924 and 1394 for undigested peptide (Abz-E-P-G-R-Q-S-R-M-R-R↓A-A-N-Tyx-A-$CONH_2$, calculated MW = 1924) and the N-terminal (NT) cleaved fragment (Abz-E-P-G-R-Q-S-R-M-R-R-OH, calculated MW = 1394), respectively, confirming cleavage at RMRR↓AA. The peak for carboxy terminal (CT) fragment (A-A-N-Tyx-A-$CONH_2$, calculated MW = 438) could not be detected because of its small size, poor ionization and possible overlapping with background matrix peaks. It was further observed that NT fragment sequentially lost one amino acid at a time from its CT leading to peaks at *m/z* 1234 (NT-Arg), 1082 (NT-Arg-Arg), 949 (NT-Met-Arg-Arg), 793 (NT-Arg-Met-Arg-Arg) and 706 (NT-Ser-Arg-Met-Arg-Arg) (Fig. 7A) suggesting the presence of carboxypeptidase Y (CpY)-like enzyme/s [28] as minor contaminant/s in rec-PC4 sample. This was further confirmed by the observation that sequential loss of CT-amino acids, one at a time, could be suppressed (Fig. 7B) by prior incubation with CpY-inhibitor, Gemsa (guanidinoethyl mercapto succinic acid) [29]. It is well known that CpY cleaves either basin or nonbasic amino acid from C-terminus of a peptide chain.

Table 1
Purification of recombinant ratPC4-FLV5 obtained from concentrate medium *Leishmania tarentolae* expression system

Sample	Total volume (ml)	Total protein (P) (μg/μl)	Total enzyme activity (E) (U/μl)	Specific activity (E/P) (U/μg)	Yield (%)	Fold purification from crude
Crude concentrate culture medium of rec-rPC4-FLV5	1.2*	2.9	13.5	4.65	100	–
100 mM NaCl pool (DEAE column)	21	0.15	14.6	97.23	108	20.9
17.5 mM arginine fraction E1-pool (Arg-column)	10	0.08	11.83	147.8	88	31.8
17.5 mM arginine fraction E2 pool (Arg-column)	9	0.065	12.65	194.6	94	41.8
20 mM arginine fraction E3 pool (Arg-column)	11	0.1	15.9	159	118	34.2

Comparison of protein content and activity profiles of various PC4 fractions following DEAE and arginine-column chromatographies with the crude sample.
Note: *, It was obtained from 60 mL of *Leishmania* culture medium; U, pmol of free AMC released per min following incubation at 37 °C with 50 μM Boc-RVRR-MCA. The data are average of three independent measurements from two separate purification experiments.

PC4-prodomain peptide inhibitor

Previously we demonstrated that the PC4-prodomain peptide, rPC4$^{101-110}$ located at the N-terminus immediately of its primary activation site (RVKR^{110}↓SL), inhibits PC4-activity with moderate efficiency [8]. However a detailed analysis about its potency and selectivity was not properly investigated. In order to enhance PC4-inhibition potency and selectivity, we have generated another peptide namely rPC4$^{101-116}$ that contains both P and P′ residues [24–30]. This peptide at 50 μM concentration significantly suppressed PC4-cleavage of Q-fertilin-α (Fig. 7C and D). When measured against the fluorogenic substrate Boc-RVRR-MCA, rPC4$^{101-116}$ also exhibited a strong competitive inhibition of PC4 with K_i ~3.8 μM as determined by Cornish-Bowden plot (Fig. 8. A). The measured IC_{50} value is ~20 μM for Boc-RVRR-MCA (50 μM). At low substrate concentration (<20 μM), this peptide showed a different interactive behavior against PC4. Thus at concentrations below 1 μM, it enhanced PC4-activity depending on concentration (by as much as ~2-fold). This activation effect was less significant for a higher substrate concentration and was not noticeable at ⩾20 μM. Similar effects were also observed with another fluorogenic substrate namely pERTKR-MCA suggesting that it is not a substrate-dependent event. Such bimodal effect of prodomain derived peptide on enzyme activity has been previously reported for PC1 enzyme, a close relative of PC4 [31]. In addition to this prodomain PC4-inhibitor, we also examined and compared the PC4-inhibitory properties of two commercially available peptide chloromethyl ketones (cmk), namely Dec-RVRR-cmk, a known general PC-inhibitor and the related Dec-RVRK-cmk. These derivatives efficiently inhibited recPC4-activity (present in E1 pool) with IC_{50} = 16.6 ± 2.5 nM and 12.2 ± 0.7 nM, respectively (Fig. 8B and C). To our knowledge, this is a first demonstration of a Lys-cmk containing peptide inhibiting a PC-enzyme.

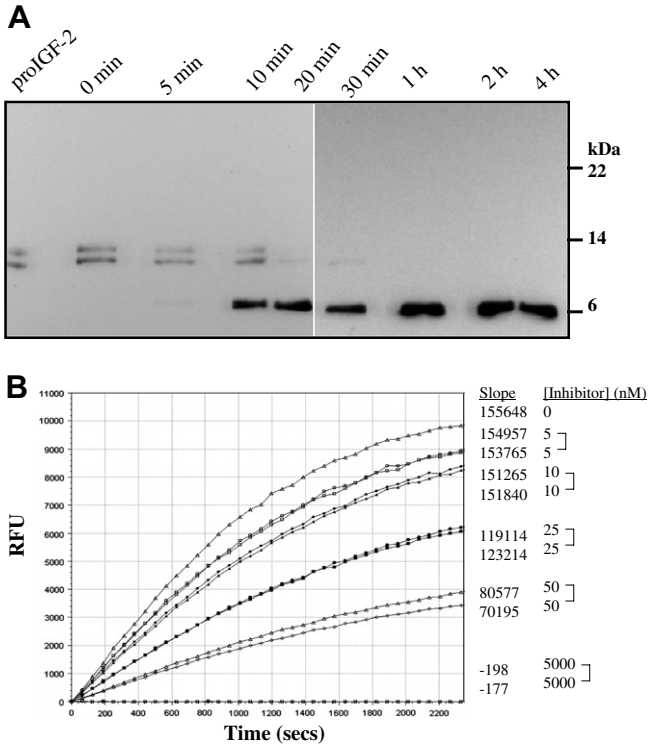


Fig. 6. (A) Western blot analyses on crude digests of recombinant proIGF-2 by purified sample (E1 fraction) of rPC4-FLV5 obtained from DEAE followed by arginine-column chromatographies. The digestion was followed at various time points ranging from 0 to 5 h using anti-IGF-2 antibody. (B) Progress curves showing inhibition of purified rPC4-activity by Dec-RVKR-cmk. Two microliters of purified PC4 (E1 fraction from arginine-column after DEAE) representing 25.65 U/mg protein (where 1 U = activity that releases 1 pmol of free AMC per min from 50 μM Boc-RVRR-MCA) was incubated with varying concentrations ranging from 5 to 5000 nM of Dec-RVKR-cmk. The activity was measured on line against the fluorogenic substrate Boc-RVRR-MCA (50 μM).

PC4 antibody

Three antibodies namely anti-V5, anti-mPC4$^{23-393}$ (Ab-1) and anti-rPC4$^{419-441}$ (Ab-2) were used in the present study for immunodetection of PC4 protein. Ab-1 and Ab-2 antibodies were able to detect 63 and/or 52/38 kDa PC4 proteins in LEXSY media whereas anti-V5 antibody failed to detect any of these bands. This suggested the loss of V5-tag in secreted PC4 protein. A comparative analysis of Western blots of E1, E2 and E3 fractions using Ab-1 and Ab-2 as primary antibodies confirmed that both commercial Ab-1 and our Ab-2 antibodies were able to recognize the enzymatically active 62 kDa PC4 protein with a high degree of efficiency (data not shown).

Discussion

Selective localization of PC4 in reproductive tissues [4–7] and its critical role in fertilization via activation of sperm surface proteins have led to an enormous interest in the study of this enzyme. As part of our ongoing program in this field, we have now produced rec-rPC4 in active soluble form with much higher specific activity (4.65 U/μg protein) using LEXSY. This is highly significant in com-

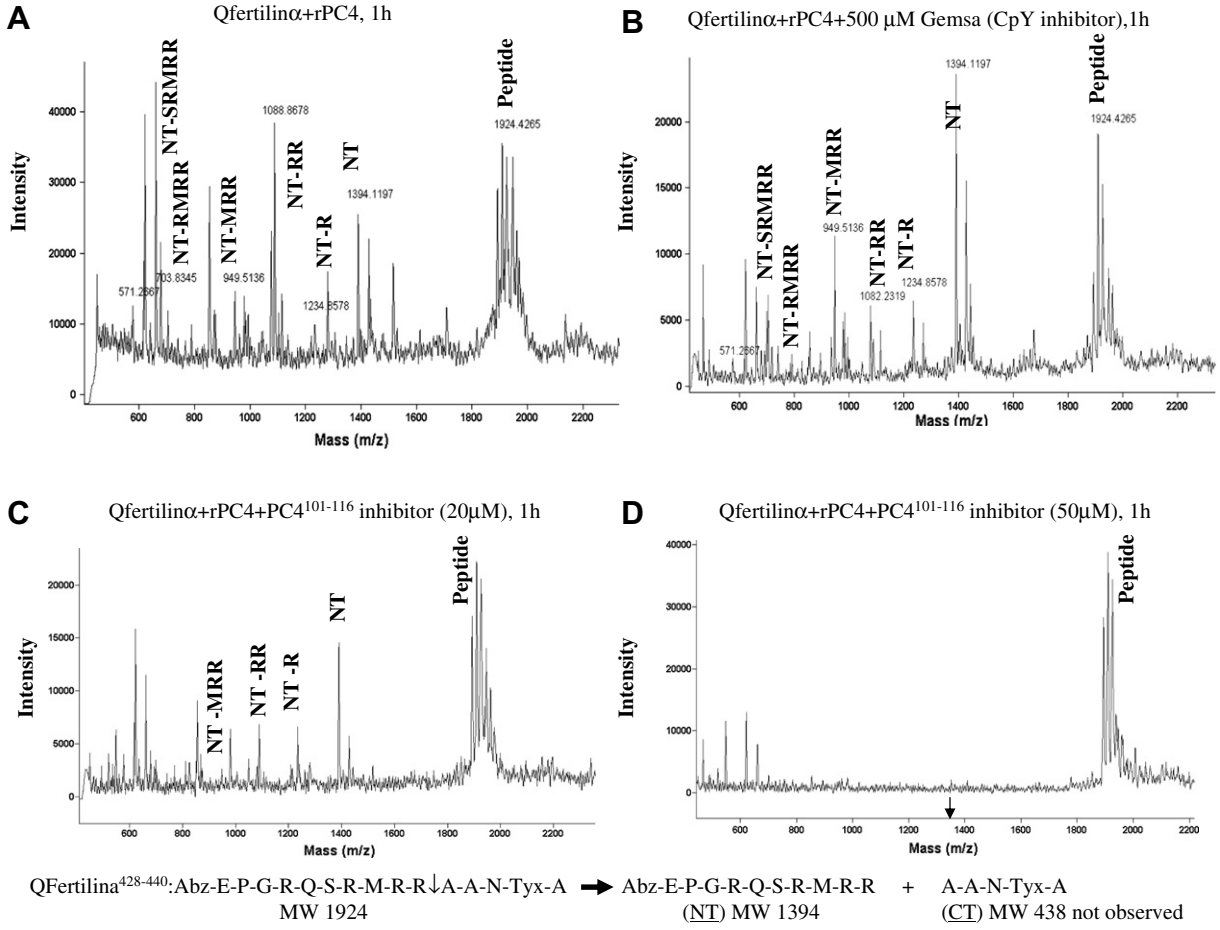


Fig. 7. MALDI-tof-mass spectra of crude digests of Q-mFertilin $\alpha^{428-440}$ (10 μg) with purified recombinant rPC4 enzyme [2 μl of 10-fold diluted of E1 fraction obtained from arginine-column with overall specific activity of 2.5 U/mg protein where U = unit of activity as defined previously] in the absence and presence of either carboxypeptidase Y inhibitor, gemsa, (500 μM) or a PC4-inhibitor, $rPC4^{101-116}$ peptide (20 and 50 μM). NT = N (Amino) terminal fragment and CT = C (Carboxy) terminal fragment. The purified PC4 sample used in this experiment consisted of mostly the active 62 kDa PC4 protein. Cleavage by PC4 yielded C-terminal (CT) and N-terminal (NT) fragments as shown at the bottom of the figure. The sequential loss of one amino acid at a time from the carboxy terminal of CT due to the action of carboxypeptidase Y (present as contaminant) leading to various fragments is also shown in the figure. All cleavages are indicated by vertical arrowheads (↓).

parison to our earlier efforts using mammalian GH4C1 [2] and Hi5 [3] cells. Moreover in this study, active rec-PC4 was purified for the first time by two-step chromatographic methods involving DEAE and arginine–sepharose columns. Purified PC4 sample showed three immunoreactive protein bands at ~62, 53 and 38 kDa positions of which, only the first exhibited a strong positive band in fluorescent in-gel zymogram, confirming its protease activity towards the fluorogenic substrate Boc-RVRR-MCA. This also suggested that 62 kDa protein must have lost the prodomain and consequently starts with the sequence ^{111}SLVV. The size 62 kDa was explained by the loss of V5-tag + a short C-terminal tail from $rPC4^{111-654}$ + V5. One possibility is that a cleavage occurred within the segment (600–654), most likely at HCGR^{617}↓TL. Such a cleavage, possibly an autocatalytic one, will lead to $rPC4^{111-617}$ (MW 55,900). Alternatively the cleavage may occur at an unknown C-terminal site by the action of a protease/s endogenous to LEXSY [26,27], thereby deleting the V5-tag. For inactive ~52 kDa PC4 band, we propose that it may correspond to $rPC4^{27-486}$ (MW = 51,534 Da) generated by autocatalytic shedding at RRR-LIR^{486}↓SL. Other autocatalytic sites such as LSYSRR^{502}↓GD or RSTLVAIR^{523}↓PL may also explain the formation of the above PC4 protein band. C-terminal truncated shed forms have been reported for transmembrane bound furin, SKI-1 as well as PC1, PC5 and PC7 [1]. Alternatively cleavage by an endogenous protease at an unknown site cannot be ruled out. Finally it is possible that the other inactive ~38 kDa PC4 protein found mainly in E2 and E3 pool may be derived from another autocatalytic shedding at RASR^{404}↓PA leading to the fragment $rPC4^{27-404}$ (MW = 42,272). Another possibility is that this band may in fact be an active $rPC4^{111-486}$ (MW = 41,312), whose activity could not be detected due to instability in our fluorescent zymogram condition. Further studies will be required to validate one or more of these propositions. The observation that a significant amount of PC4 protein remained bound to cell membrane supported the earlier notion that PC4 despite lacking a transmembrane domain is associated with cell membrane possibly via GPI (glycosyl phosphatidylinositol) anchor [9].

Our purified LEXSY rec-PC4 exhibited protease activity towards synthetic fluorogenic MCA and IQF-peptides as well as pro-proteins such as IGF-2, despite the presence of carboxypeptidase Y (CpY) as a contaminant. We showed that this exopeptidase activity can be easily suppressed by a specific CpY-inhibitor, gemsa. We showed that our purified rec-PC4 efficiently cleaved 14 kDa pro-IGF-2 yielding 7 kDa mature form [9]. No additional cleavage was noted even upon prolonged digestion confirming that proIGF-2 in reproductive tissues is a natural substrate of PC4. Presence of CpY activity is not uncommon in many mammalian and protozoan expression systems including LEXSY [2,3]. In future, studies could be directed to completely remove CpY activity from the purified PC4-activity.

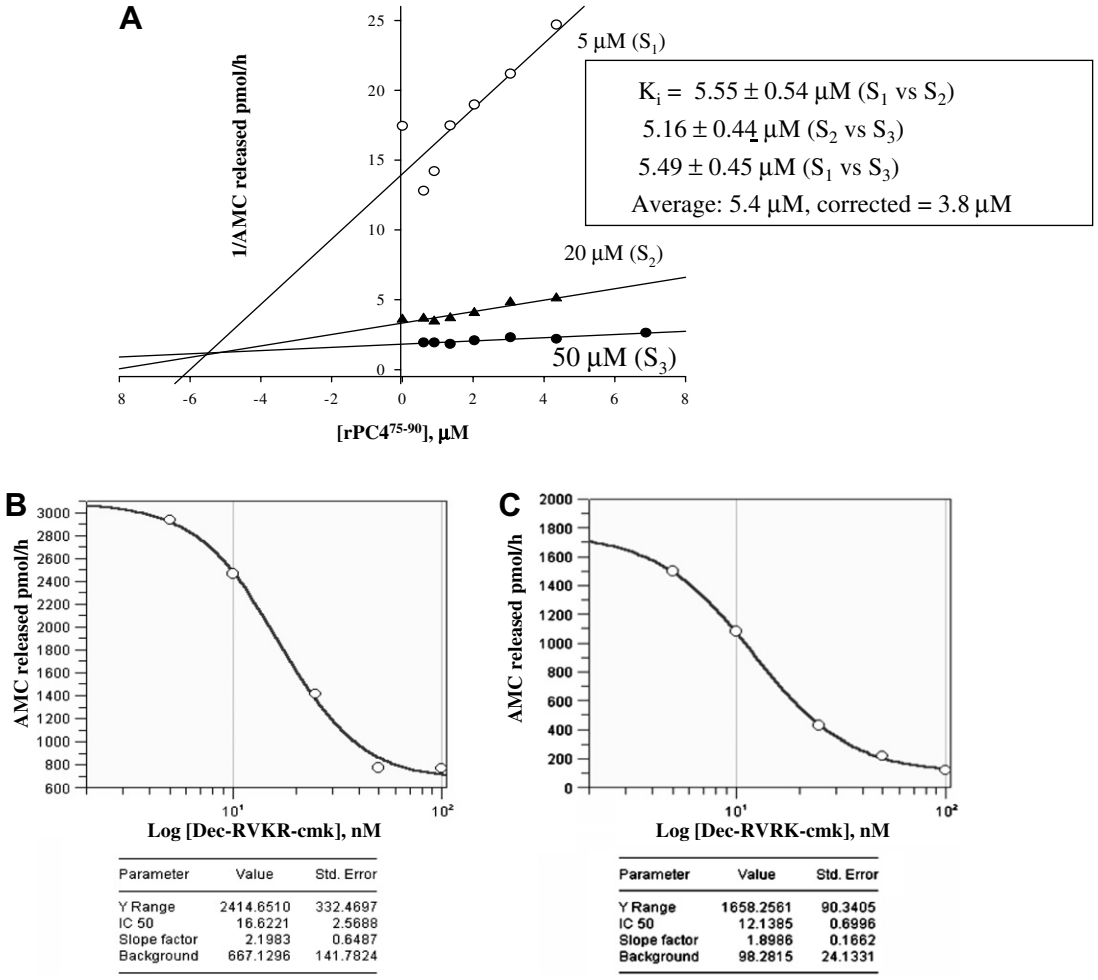


Fig. 8. (A) Graphic presentation of Cornish–Bowden plot (1/V vs inhibitor concentration) showing inhibition of purified rec-rPC4-activity by $rPC4^{101-116}$ peptide. Inhibition constant, K_i, was measured by using three different concentrations (5, 20 and 50 μM) of PC4-substrate Boc-RVRR-MCA as indicated in the figure. Raw fluorescence units (RFU) released per hour were measured with wavelengths fixed at λ_{ex} = 360 and λ_{em} = 470 nm and the data transformed into pmol free AMC released per hour by using standard curve. Curves were best fitted as described in Experimental Procedures. (B and C) Inhibition of rec-rPC4-activity by peptidyl chloromethyl ketones (cmks) at various concentration levels. Sigmoidal graphs obtained from plot of enzyme activity (measured as pmol free AMC released per hour from 50 μM Boc-RVRR-MCA) of purified rec-rPC4 vs logarithm of concentration of PC4 inhibitor namely Dec-RVRR-cmk or Dec-RVRK-MCA. Measured IC_{50} values obtained from the graphs and corresponding standard deviations are displayed in the figures.

In this study we also reported the production of a new polyclonal PC4-Ab developed against the C-terminal $rPC4^{419-441}$ domain, which like the commercial Ab-1 antibody was able to detect efficiently both 62 and 53 kDa PC4 bands. Currently development of a third PC4-Ab against a conserved domain is in progress. Such an antibody is expected to be useful for detection of endogenous PC4 in tissue and biological samples irrespective of species of origin.

In light of increasing interest in PC4-inhibitors for clinical and therapeutic reasons, we have developed small PC4-inhibitors in this study. Our data showed that Dec-RVRR-cmk and Dec-RVRK-cmk both inhibited PC4-activity with a high degree of potency (IC_{50} ~12–16 nM range). This suggested that in the context of this tetrapeptide sequence, PC4 recognizes a Lys residue at P1 position as efficiently as the Arg residue. However it will be of great interest to examine and compare the inhibitory effects of these peptide-cmks towards other PC-enzymes. Previous studies indicate that PCs including PC4 cleave protein bonds only at the C-terminus to Arg but not to Lys [1,2]. Therefore in this regard, the current study provided additional information about the substrate specificity of the enzyme. Another aspect of this study is the demonstration that the prodomain peptide $rPC4^{101-116}$ encompassing the primary cleavage site is a strong competitive inhibitor of PC4 enzyme with K_i ~3.8 μM when measured against Boc-RVRR-MCA. However at very low concentration, it actually activated PC4 slightly. This bimodal behavior may be explained by the fact that the peptide first behaved as a substrate to PC4 enzyme and then exerted its inhibitory property possibly via the cleaved fragments. The above PC4-prodomain peptide also suppressed PC4-mediated cleavage of model synthetic IQF-peptides derived from ADAMs-1, 2 and 5 in a dose-dependent manner. Further studies need to be carried out to study the effect of this inhibitor on the processing of full length forms of these proteins owing to their crucial role in sperm–egg interaction and fertilization. In fact our very recent data revealed that this peptide, in fact, can prevent PC4 processing of ADAM-2 protein isolated from mouse sperm. Furthermore its dose-dependent inhibitory action on sperm–egg interaction and in vitro fertilization in mouse model has also been demonstrated (manuscript in preparation). Availability of nearly pure and active soluble rec-PC4, its synthetic inhibitors and a polyclonal antibody developed against its C-terminal domain will be extremely valuable for further biological study and characterization of PC4 enzyme. In addi-

tion it will also help to identify and/or confirm its proposed physiological substrates. This includes not only the ADAM proteins and IGFs but also the recently discovered epididymal antimicrobial peptides such as HE (human epididymus) and SPAG (sperm antigen) proteins of defensin family [32] found in human epididymal duct. Preliminary studies in our laboratory revealed that these proteins may be processed by PC4 enzyme to generate sperm binding antimicrobial peptides but the exact cleavage site and the enzyme responsible have yet not been identified. Based on their expression sites and cleavage motifs, PC4 appears to be a good candidate. The present study further indicated the role of PC4-activity in the maturation of sperm surface proteins such as ADAMs and IGF-2 and demonstrates the potential of PC4-inhibitors as non-hormonal contraceptive and sperm protective agents against infection. Our study will further draw attention to increasing research in this important field of reproductive biology with PC4 as the central molecule. The regulation of activity of this enzyme is an important balancing factor for healthy conception.

Acknowledgments

We thank Dr. N. Tanphaichitr of OHRI for critical reading of the manuscript and valuable comments. We also thank Dr. S. Basak and Andrew Chen for synthesis of IQF peptides, Francine Sirois and Charles Gyamera-Acheampong for technical assistance for PC4 production using the *Leishmania* expression system. This work was funded by Canadian Institutes of Health Research Grant to AB (MOP-69093).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2008.03.013.

References

[1] N.G. Seidah, M. Chrétien, Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides, Brain Res. 848 (1999) 45–62.
[2] A. Basak, B.B. Touré, C. Lazure, M. Mbikay, M. Chrétien, N.G. Seidah, Enzymic characterization in vitro of recombinant proprotein convertase PC4, Biochem. J. 343 (1999) 29–37.
[3] S. Basak, M. Chrétien, M. Mbikay, A. Basak, In vitro elucidation of substrate specificity and bioassay of proprotein convertase 4 using intramolecularly quenched fluorogenic peptides, Biochem. J. 380 (2004) 505–514.
[4] N.G. Seidah, R. Day, J. Hamelin, A. Gaspar, M.W. Collard, M. Chrétien, Testicular expression of PC4 in the rat: molecular diversity of a novel germ cell-specific Kex2/subtilisin-like proprotein convertase, Mol. Endocrinol. 6 (1992) 1559–1570.
[5] S. Torii, T. Yamagishi, K. Murakami, K. Nakayama, Localization of Kex2-like processing endoproteases, furin and PC4, within mouse testis by in situ hybridization, FEBS Lett. 316 (1993) 12–16.
[6] H. Tadros, M. Chrétien, M. Mbikay, The testicular germ-cell protease PC4 is also expressed in macrophage-like cells of the ovary, J. Reprod. Immunol. 49 (2001) 133–152.
[7] C. Gyamera-Acheampong, J. Tantibhedhangakul, H. Tadros, F. Sirois, W. Weerachatyanukul, J.W. van de Loo, R.M. Pelletier, N. Tanphaichitr, M. Mbikay, Proprotein convertase 4 (PC4) is located on the acrosomal plasma membrane and its deficiency renders spermatozoa less efficient at egg-binding, Biol. Reprod. 74 (2006) 666–673.
[8] Q. Qiu, A. Basak, M. Mbikay, B.K. Tsang, A. Gruslin, ProIGF-II processing in human placenta: the expression and role of proprotein convertase PC4, Proc. Natl. Acad. Sci. USA 102 (2005) 11047–11052.
[9] M. Mbikay, H. Tadros, N. Ishida, C.P. Lerner, E. De Lamirande, A. Chen, M. El-Alfy, Y. Clermont, N.G. Seidah, M. Chrétien, C. Gagnon, E.M. Simpson, Impaired fertility in mice deficient for the testicular germ-cell protease PC4, Proc. Natl. Acad. Sci. USA 94 (1997) 6842–6846.
[10] N. Scamuffa, F. Calvo, M. Chretien, N.G. Seidah, A.M. Khatib, Proprotein convertases: lessons from knockouts, FASEB J. 20 (2006) 1954–1963.
[11] M. Li, M. Mbikay, K. Nakayama, A. Miyata, A. Arimura, Prohormone convertase PC4 processes the precursor of PACAP in the testis, Ann. NY Acad. Sci. 921 (2000) 333–339.
[12] S.J. Duguay, W.M. Milewski, B.D. Young, K. Nakayama, D.F. Steiner, Processing of wild-type and mutant proinsulin-like growth factor-IA by subtilisin-related proprotein convertases, J. Biol. Chem. 272 (1997) 6663–6670.
[13] P. Primakoff, D.G. Myles, The ADAM gene family: surface proteins with adhesion and protease activity, Trends Genet. 16 (2000) 83–87.
[14] C.P. Blobel, Functional processing of fertilin: evidence for a critical role of proteolysis in sperm maturation and activation, Rev. Reprod. 5 (2000) 75–83.
[15] S.J. Waters, J.M. White, Biochemical and molecular characterization of bovine fertilin α and β (ADAM-1 and ADAM-2): a candidate sperm–egg binding/fusion complex, Biol. Reprod. 56 (1997) 1245–1254.
[16] E. Kim, H. Nishimura, S. Iwase, K. Yamagata, S. Kashiwabara, T. Baba, Synthesis, processing, and subcellular localization of mouse ADAM3 during spermatogenesis and epididymal sperm transport, J. Reprod. Dev. 50 (2004) 571–578.
[17] T.G. Wolfsberg, P.D. Straight, R.L. Gerena, A.P. Huovila, P. Primakoff, D.G. Myles, J.M. White, ADAM, a widely distributed and developmentally regulated gene family encoding membrane proteins with a disintegrin and metalloprotease domain, Dev. Biol. 169 (1995) 378–383.
[18] I.M. Adham, K. Nayernia, W. Engel, Spermatozoa lacking acrosin protein show delayed fertilization, Mol. Reprod. Dev. 46 (1997) 370–376.
[19] S. Kushnir, K. Gase, R. Breitling, K. Alexandrov, Development of an inducible protein expression system based on the protozoan host *Leishmania tarentolae*, Protein Expr. Purif. 42 (2005) 37–46.
[20] A.J. Yakovich, F.L. Ragone, J.D. Alfonzo, D.L. Sackett, K.A. Werbovetz, *Leishmania tarentolae*: purification and characterization of tubulin and its suitability for antileishmanial drug screening, Fertil. Steril. 83 (2005) 1780–1790.
[21] A. Niculae, P. Bayer, I. Cirstea, T. Bergbrede, R. Pietrucha, M. Gruen, R. Breitling, K. Alexandrov, Isotopic labeling of recombinant proteins expressed in the protozoan host *Leishmania tarentolae*, Protein Expr. Purif. 48 (2006) 167–172.
[22] M.R. Groseclose, M. Andersson, W.M. Hardesty, R.M. Caprioli, Identification of proteins directly from tissue: in situ tryptic digestions coupled with imaging mass spectrometry, J. Mass Spectrom. 42 (2007) 254–262.
[23] S. Yasothoensrikul, V.Y. Hook, Detection of proteolytic activity by fluorescent zymogram in gel assays, Biotechniques 28 (2000) 1166–1173.
[24] A. Basak, C. Lazure, Synthetic peptides of prosegments of proprotein convertases PC1 and furin are inhibitors of both convertases with some selectivity, Biochem. J. 373 (2003) 231–239.
[25] A. Basak, A. Boudreault, A. Chen, M. Chrétien, N.G. Seidah, C. Lazure, Application of the multiple antigenic peptides (MAP) strategy to the production of prohormone convertases antibodies: synthesis, characterization and use of 8-branched immunogenic peptides, J. Pept. Sci. 1 (1995) 385–395.
[26] R. Dey, J. Bhattacharya, S.C. Datta, Calcium-dependent proteolytic activity of a cysteine protease caldonopain is detected during *Leishmania* infection, Mol. Cell. Biochem. 281 (2006) 27–33.
[27] R. Etges, Identification of a surface metalloproteinase on 13 species of *Leishmania* isolated from humans, *Crithidia fasciculata*, and *Herpetomonas samuelpessoai*, Acta Trop. 50 (1992) 205–217.
[28] S.E. Reznik, L.D. Fricker, Carboxypeptidases from A to z: implications in embryonic development and Wnt binding, Cell. Mol. Life Sci. 58 (2001) 1790–1804.
[29] B. Przewlocka, M. Dziedzicka, J. Silberring, W. Lason, Analgesic and convulsant effects of guanidinoethylmercaptosuccinic acid (GEMSA)—a potent enkephalin convertase inhibitor, Neuropeptides 8 (1986) 359–365.
[30] M. Zhong, J.S. Munzer, A. Basak, S. Benjannet, S.J. Mowla, E. Decroly, M. Chrétien, N.G. Seidah, The prosegments of Furin and PC7 as potent inhibitors of proprotein convertases: in vitro and ex vivo assessment of their specificity and selectivity, J. Biol. Chem. 274 (1999) 33913–33920.
[31] N. Rabah, D. Gauthier, J.D. Dikeakos, T.L. Reudelhuber, C. Lazure, The C-terminal region of the proprotein convertase 1/3 (PC1/3) exerts a bimodal regulation of the enzyme activity in vitro, FEBS J. 274 (2007) 3482–3491.
[32] H.H. von Horsten, B. Schafer, C. Kirchhoff, SPAG11/isoform HE2C, an atypical anionic beta-defensin-like peptide, Peptides 25 (2004) 1223–1233.