Iranian Journal of Pharmaceutical Sciences
Winter 2006: 2(1): 11-22
www.ijps.ir

Original Article

Bioengineered Peptides Based on α1-PDX Structure as Inhibitors of Furin: Design, Synthesis and Comparative Efficacy

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Abstract

Furin is a Ca^{2+} -dependent serine protease which cleaves proprotein substrates at the Arg-Xaa-(Lys/Arg)-Arg ↓ site to generate biologically active proteins. Furin's critical role in many cellular events associated with health disorders such as HIV, SARS, anthrax, and influenza as well as cancer has made inhibitors of this enzyme as therapeutic targets. To this date, the most potent inhibitor of furin is the bioengineered serpin (serine protease inhibitors) protein namely α1-PDX. It was already demonstrated that the reactive site loop (RSL) of all serpins are prime interactive domains responsible for their protease inhibitory function. Therefore, the objective of the present study was to develop small peptides with the RSL structure of serpin α1-PDX, as inhibitors of furin activity. Fifteen peptides were designed from reactive site loop structure of α1-PDX (sequences 367-394) with different mutations in this site, and were synthesized using a solid-phase peptide synthesizer, and characterized by MALDI-tof mass spectrometry and amino acid analysis. The inhibitory effects of the designed peptides against furin activity were evaluated by spectroflourometry using QVEGF-C [Abz-QVHSIIRRßSLP-Y(NO_2)-A-$CONH_2$, Abz = 2-amino benzoic acid and Y(NO_2) = 3-nitro tyrosine] as substrate. The results showed that all of the designed peptides inhibit furin activity with different efficacies in a time and concentration dependent pattern. Peptides containing His or straight alkyl side chain amino acids in positions P2, P3, P6 and P8 have higher efficacy for blocking furin activity in comparison with peptides containing Arg or Lys in that position. The study further revealed that the peptides inactivate furin in a slow tight binding pattern. Our study provides an alternate strategy for development of efficient peptide-based inhibitors of proprotein convertases such as furin.

Keywords: α1-PDX; Furin; Proprotein convertase; Protease inhibitor; Serpin.
Received: September 28, 2005; *Accepted:* November 2, 2005

1. Introduction

The discovery of mammalian subtilases, the proprotein convertases (PCs), in 1990

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came as a culmination of a prolonged and sustained effort to hunt for enzyme/s responsible for proteolytic maturation of inactive precursor proteins [1-3]. Since then seven PCs namely PC1/3, PC2, PC4, PC5/6, PC7/8/LPC, furin/PACE (paired-basic-amino acid-converting enzyme) and PACE4 have been discovered that cleave at the carboxyl terminal of the consensus Arg/Lys-X-X/Lys-Arg↓ site [4]. Proteolytic processing of larger inactive precursor proteins into smaller functionally bioactive forms is an important cellular event implicated in both normal and abnormal situations. Because of this role, proteolytic enzymes remain in the forefront as important therapeutic targets for intervention of many diseases and metabolic disorders, even though several of these proteases are also linked to normal development and important physiological functions [1-4].

Furin is the first and so far the best characterized enzyme of the mammalian subtilisin-like family of Ca^{+2}-dependent cellular endoproteases. As well as activating pathogenic agents, furin has an essential role in embryogenesis, and catalyses the maturation of a diverse collection of proprotein substrates. These range from growth factors and receptors to extracellular-matrix proteins and even other protease systems that control disease. Furin's crucial role in so many different cellular events, and in diseases ranging from anthrax and bird flu to cancer, dementia and Ebola fever, has caused researchers to re-evaluate it [5-8].

The development of potent and specific protease inhibitors became an important research topic in drug design and therapeutic applications. Already a number of inhibitors directed against furin have been described in the literature for treatment of conditions such as viral infections like AIDS, inflammatory diseases such as chronic obstructive pulmonary disorder, prostate cancer and others [9-12]. Despite several reports, not much significant success has been achieved in designing potent small compound inhibitors of furin, that are more attractive as therapeutic

Table 1. List of various peptides derived from the amino acid sequence of reactive site loop of Alpha 1-PDX containing the furin cleavage motif.

Peptide I	Cys-367KGTEAAGAMFLE**RIPR**↓ fSIPPEVKFNKPF394-Cys
Peptide II	Cys-367KGTEAAGAMFLE**KIPR**↓ fSIPPEVKFNKPF394-Cys
Peptide III	Cys-367KGTEAAGAMFLE**HIPR**↓ fSIPPEVKFNKPF394-Cys
Peptide IV	Cys-367KGTEAAGAMFLE**RIRR**↓ fSIPPEVKFNKPF394-Cys
Peptide V	Cys-367KGTEAAGAMFLE**RIKR**↓ fSIPPEVKFNKPF394-Cys
Peptide VI	Cys-367KGTEAAGAMFLE**RIHR**↓ fSIPPEVKFNKPF394-Cys
Peptide VII	Cys-367KGTEAAGAMFLE**RVPR**↓ fSIPPEVKFNKPF394-Cys
Peptide VIII	Cys-367KGTEAAGAMFLE**RLPR**↓ fSIPPEVKFNKPF394-Cys
Peptide IX	Cys-367KGTEAAGAMFLE**RFPR**↓ fSIPPEVKFNKPF394-Cys
Peptide X	Cys-367KGTEAAGAMFLE**RWPR**↓ fSIPPEVKFNKPF394-Cys
Peptide XI	Cys-367KGTEAAGAMF**R**E**RIPR**↓ fSIPPEVKFNKPF394-Cys
Peptide XII	Cys-367KGTEAAGAMF**K**E**RIPR**↓ fSIPPEVKFNKPF394-Cys
Peptide XIII	Cys-367KGTEAAGAMF**H**E**RIPR**↓ fSIPPEVKFNKPF394-Cys
Peptide XIV	Cys-367KGTEAAGAMF**R**E**KIPR**↓ fSIPPEVKFNKPF394-Cys
Peptide XV	Cys-367KGTEAAGA**K**F**K**E**RIPR**↓ fSIPPEVKFNKPF394-Cys

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A

B

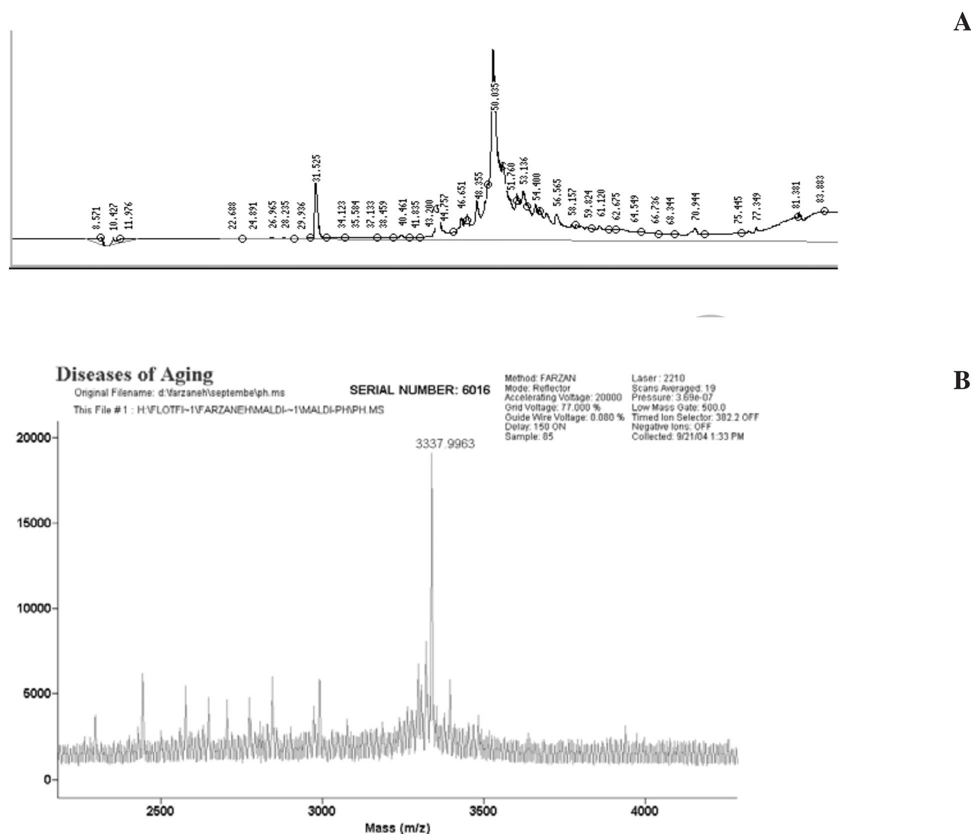

Figure 1. RP-HPLC chromatograms of crude peptide (VIII) (A) and its corresponding MALDI mass spectra (B).

agents because of their stability and accessibility. In the present study, we report the development of peptide molecule inhibitors of furin consisting of the RSL structure of a1-PDX with different mutations in P1 to P8 positions that block furin activity with different potencies. These findings were based on *in vitro* experiments using small peptide and physiological protein substrates.

2. Materials and methods

2.1. Materials

All Fmoc (fluoren-9-yl methoxycarbonyl) amino acids (L-configuration), the coupling agents and organic solvents were purchased from PE Applied Biosystems (Framingham, MA, USA), Calbiochem–Novabiochem (San Diego, CA, USA), Chem-Impex International (Wood Dale, IL, USA) and Aldrich Chemical Company (Milwaukee, WI, USA). Recombinant furin enzyme and its substrate Boc-RVRR-MCA (MCA = 4-methyl coumaryl-7-amide) used for kinetic study were purchased from New England BioLabs Inc. (MA, USA) and Peptides International (Louisville, KY, USA), respectively. The intramolecularly quenched fluorogenic substrate,QVEGF-C[Abz-QVHSIIRR^{227} ßSLP-Y(NO_2)-A-$CONH_2$, MW 1701, Abz = 2-amino benzoic acid and Y(NO_2) = 3-nitro tyrosine] used in the present study as a furin substrate [13]. This peptide was derived from the physiological processing site RR^{227}ßSL of VEGF-C. Acetonitrile (ACN), 4- hydroxycinnamic acid (CHCA), HCl, triflouro ethanol (TFE), and all other reagents were purchased from Sigma-Aldrich Company (Milwaukee, MI, USA).

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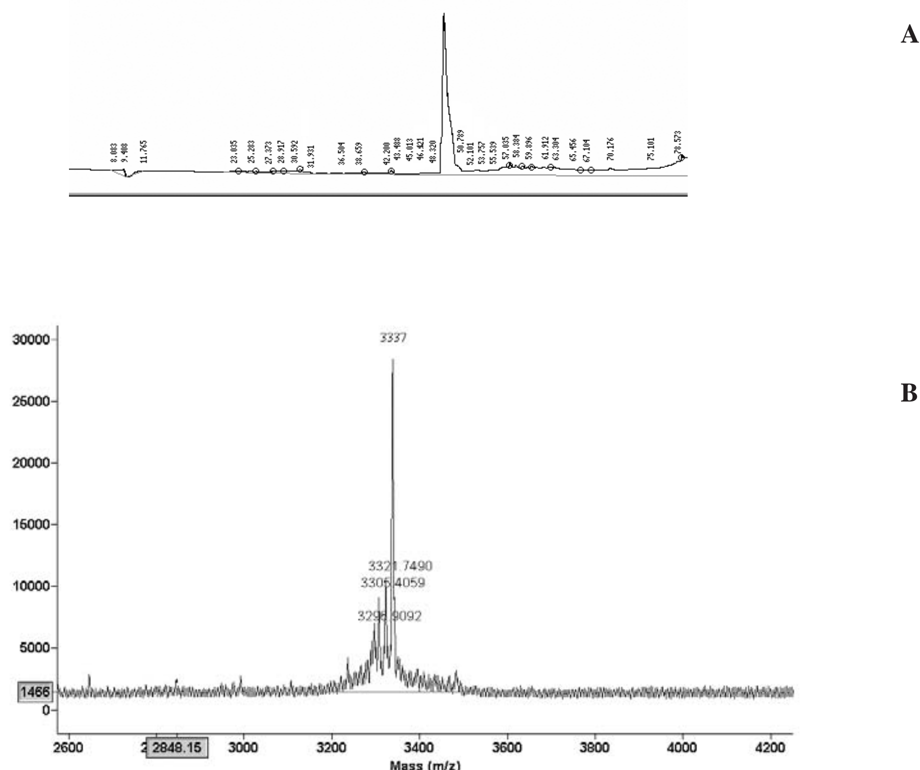

Figure 2. RP-HPLC chromatograms of pure peptide (VIII) (A) and its corresponding MALDI mass spectra (B).

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2.2. Peptide synthesis

All peptides were synthesized as C-terminal amides on an automated solid-phase peptide synthesizer (Pioneer, PE-Perseptive Biosytems Inc., Framingham, MA, USA), following O-hexafluoro-phospho-[7-azabenzotriazol-1-yl]-N,N,N',N'-tetramethyluronim (HATU)/di-isopropylethylamine (DIEA)-mediated Fmoc chemistry using unloaded PAL-PEG (polyamino linker polyethylene glycol) resin. The following amino acid side chain protecting groups were used: *t*-butyloxycarbonyl (Boc) for Lys; 2, 2, 4, 6, 7-pentamethyl dihydrobenzo furan-5-sulphonyl (Pbf) for Arg; *t*-butyl for Ser, Thr, Asp and Tyr and trityl for His, Asn and Gln. A deprotection cocktail (Reagent B) containing TFA, phenol, water and TIPS (triisopropylsilane) was used and added to the peptide-bound resin (5 ml) [13]. After 3 h of treatment at the room temperature, the peptide was recovered as described [13].

2.3. RP-HPLC protocol for peptide purification

All peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC, Rainin Dynamax) using a semi-preparative column (CSC Exsil, C_{18}, 25 cm×1.0 cm, Chromatography Specialty Co., St-Laurent, Quebec, Canada). The buffer system consisted of an aqueous 0.1% (v/v) trifluoroacetic acid solution and an organic phase of acetonitrile also containing 0.1% (v/v) trifluoroacetic acid. The elution was carried out using a linear gradient from 15-60% organic phase in 105 min. following a 10 min. isocratic step at 100% aqueous phase (gradient A, semipreparative run) or from 0-60% organic phase in 60 min. (gradient A, for analytical run). The flow rate was adjusted to 2.0 or 1.0 ml/min., respectively, and the separation was monitored by UV absorbance at 230 nm.

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Table 2. Amino acid analysis of peptide VIII (n = 3).

No	Peak name	Retention times (min.)	Height	Area	Relative area	Amount (µmol/ml)	Mean # of residue per molecule	Known observed ± SD
1	Arg	1.70	135.674	20.253	3	312.462	2	2.22 ± 0.13
2	Lys	3.52	134.465	36.32	5.38	413.185	3	2.95 ± 0.19
3	Nor	4.18	19.612	6.751	1			
4	Ala	6.58	52.148	25.113	3.72	389.609	3	3.13 ± 0.09
5	Thr	7.03	40.662	11.949	1.77	107.934	1	1.12 ± 0.11
6	Gly	7.83	38.529	22.008	3.26	273.705	2	1.95 ± 0.18
7	Val	9.22	11.222	15.257	2.26	154.459	1	1.10 ± 0.12
8	Ser	11.03	37.296	14.649	2.17	176.279	1	1.25 ± 0.05
9	Pro	11.90	67.566	37.805	5.6	608.602	4	4.34 ± 0.25
10	Ile	15.70	6.739	14.244	2.11	131.092	1	0.93 ± 0.07
11	Leu	17.28	12.066	19.645	2.91	272.134	2	1.94 ± 0.13
12	Met	18.45	22.736	12.151	1.8	140.100	1	1.00 ± 0.06
13	n.a.	24.18						
14	Phe	28.17	91.399	33.822	5.01	529.136	4	3.06 ± 0.08
15	Glu	29.33	15.253	25.046	3.71	432.249	3	3.08 ± 0.11
16	Asn	29.70	10.381	9.181	1.36	176.561	1	1.25 ± 0.09
17	n.a.	31.08						
18	**Total**		**695.748**	**304.194**	**45.06**	**4190.791**		

2.4. Amino acid analysis

Quantitative amino acid analyses were performed following 24 h hydrolysis in 6 N HCl in a sealed tube at 110 °C *in vacuo* using the ion chromatography system (ICS-2500, Dionex, Oakville, Canada) equipped with conductance detector [14]. Norluocin was used as internal standard.

The quantity of each residue in the peptide molecules was calculated using the below procedure. First of all the molecular weight of each peptide was divided by 112 (approximate average residue weight) to estimate total residue per molecule. After that the calculated µmol of all amino acids (according to the obtained relative area from ion exchange chromatograms as well as calibration equation) were sum totaled and divided by the calculated total residues per molecule to estimate µmol of peptide analyzed. µmol of each amino acid was then divided by µmol of peptide analyzed to obtain residues per peptide molecule [14].

2.5. Mass spectral analysis

The identity of each peptide was fully confirmed by matrix-assisted laser-desorption ionization-time of-flight (MALDI-TOF) mass spectrometry (Voyageur-DE Pro, PE-Biosystems Inc., Framingham, MA, USA) or surface enhanced laser desorption ionization time of flight (SELDI-TOF) (Ciphergen, CA, USA) on gold chips (http://www.ciphergen.com) using 4-hydroxycinnamic acid or 1, 2 dihydroxy benzoic acid as energy absorbing matrix [15].

2.6. Enzyme assay and kinetic evaluation

All *in vitro* enzyme studies were performed using initial rate and/or end-time assays at room temperature in a final volume of 100 μl in 96-well flat-bottom black plates (Microfluor, Dynatec, USA). The buffer consisted of 25 mM Mes, 25 mM Tris and 2.5 mM $CaCl_2$ (pH 7.4). Assays were performed using Q-VEGF-C in the concentration of 10 μM. The release of highly fluorescent 2-amino benzoic acid (Abz) containing N-terminal fragment from QVEGF-C were monitored by a spectrofluorometer instrument (Perkin Elmer model LS50B) at excitation and emission wavelengths of 370 nm/460 nm. Recombinant furin was pre-incubated for 30 min at the room 25 °C with various concentrations of the mini PDX peptide. The fluorogenic substrate

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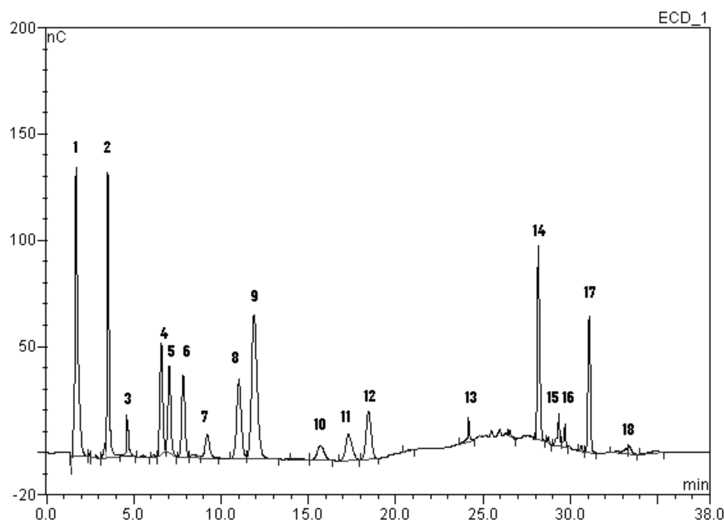

Figure 3. Ion exchange chromatogram for peptide VIII.

QVEGF-C was then added, and the released Abz-containing peptide was measured [16]. For measurement of IC_{50} (the concentration necessary to achieve 50% inhibition of the enzymic activity) values, the inhibitor concentrations were varied over a range wide enough to yield residual activities of 25-75% of the control value [16, 17].

3. Results and discussion

3.1. Design of inhibitor peptides

Furin's critical role in many cellular events associated with infections of HIV, SARS, Ebola, anthrax, and influenza as well as cancer, neurological dementia like Alzheimer's disease has made this enzyme a therapeutic target [18-20]. So far, several furin inhibitors have been reported that are

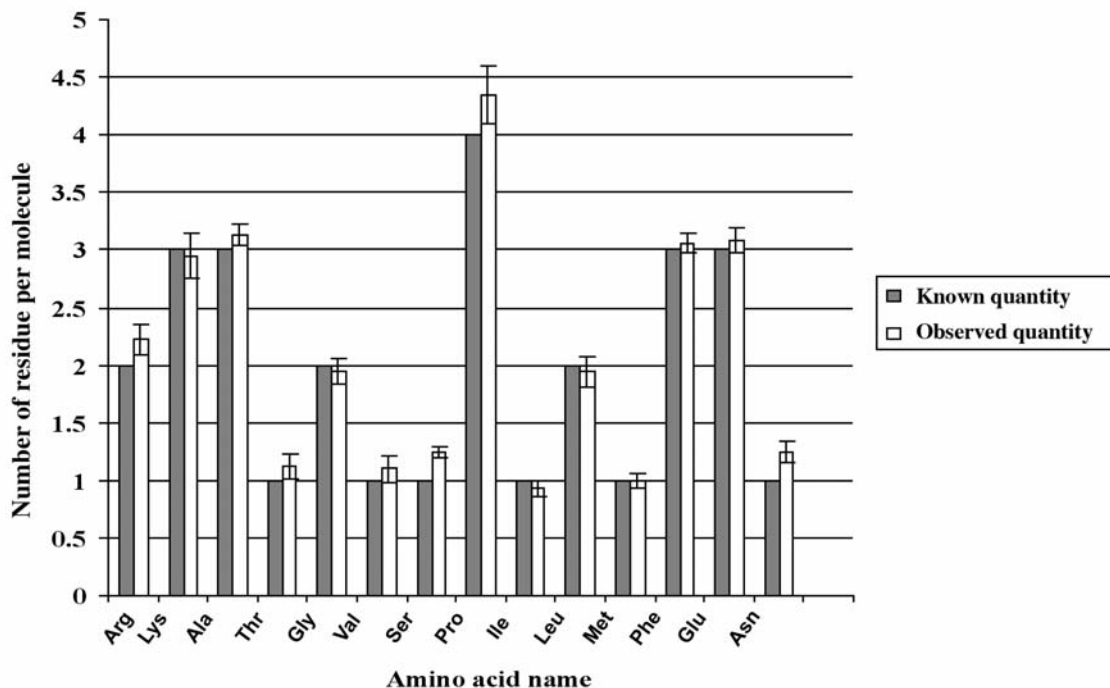

Figure 4. Bar graphs based on amino acid analysis of hydrolyzed and derivative peptide VIII.

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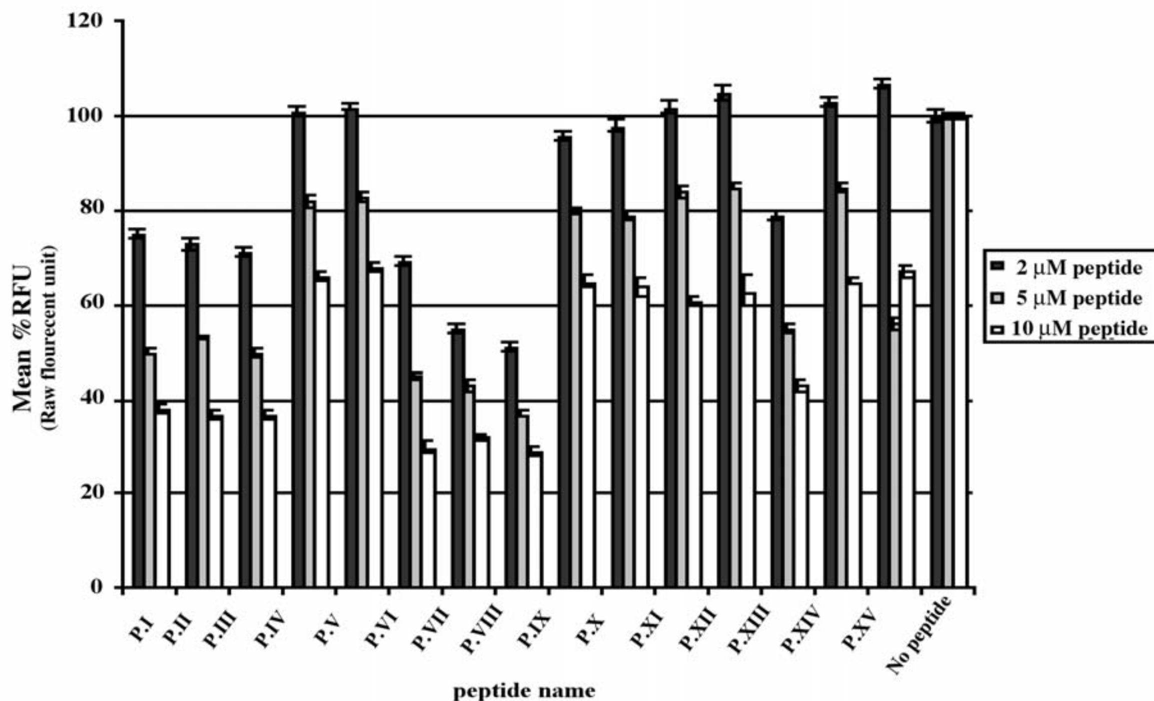

Figure 5. Bar graphs based on stop time assay to evaluate the effect of the designed peptides against furin activity after 10 minutes incubation of the digestion mixture, as measured with Q-VEGF-C (10 mcM) as a substrate (n = 3).

either macromolecular proteins or small molecule peptides to non peptides [9]. Among them the most potent is the bioengineered serpin protein namely a1-PDX with IC_{50} value of 0.6 nM [21]. It was already demonstrated that RSL (reactive site loop) of α1-PDX and also almost all of other members of serpin (serine proteinase inhibitors) family are prime interactive domains responsible for their protease inhibitory function [22, 23]. To find an optimum region of α1-PDX to design small inhibitors against furin, alignment of amino acid sequence of the most important members of serpin family, namely α1-antithrypsin, Antithrombin, C1-inhibitor, PAI (plasminogen activator inhibitor), d-SPN4 (Drosophila), ovalbumin and antichymotrypsin, within their reactive site loop (RSL) and ranking regions were performed using SIM alignment tool available at http://us.expasy.org/tools/sim-prot.html. The results of alignment indicate a resemblance of types of amino acids present at various P and P' positions as there are near conserved alkyl side chain containing hydrophobic amino acids at P_6, P_5, P_4 and P'_2 positions. Also they all contain negatively charged Glu and/or Pro residues at positions between P'_3-P'_6. Thus based on these resemblances and the hypotheses of RSL (located between sites 350-394) significant role in furin inhibition, a 30-mer peptide from residue (367-394) was designed and to study the effect of different residue, several mutations in the designed peptide was made to obtain the best choice for interacting with furin. Table 1 lists the sequences of the designed peptides.

3.2. Synthesis and characterization of the designed peptides

The designed peptides were synthesized using Fmoc protected amino acid residues by an automated peptide synthesizer machine. Following synthesis and deprotection of the peptides from PEG-PS (Poly Ethylene Glycol-Poly Styrene) resins. Peptides were lyophilized and then purified using RP-HPLC.

Peaks eluting at R_t = 51.721, 51.222, 49.631, 51.044, 51.531, 50.101, 49.361, 50.035, 49.165, 49.996, 46.339, 45.671, 46.256, 45.931, 44.259 min., respectively for

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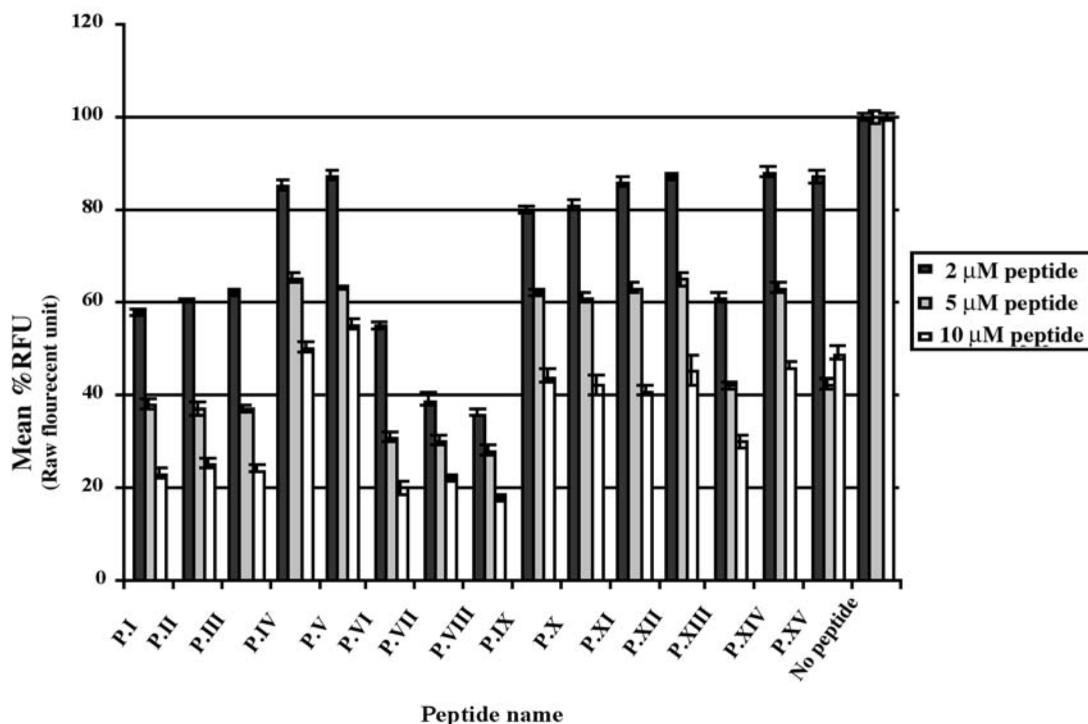

Figure 6. Bar graphs based on stop time assay to evaluate the effect of the designed peptides against furin activity after 30 min. incubation of the digestion mixture, as measured with Q-VEGF-C (10 μM) as a substrate (n = 3).

peptides I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XIV, XV, were collected and further analyzed by analytical RP-HPLC to confirm their homogeneities.

MALDI-tof mass spectra of the pure compounds showed single peaks at m/z 3337.3900, 3309.7930, 3316.7117, 3398.6922, 3369.1843, 3378.2460, 3323.6539, 3340.0908, 3374.1002, 3410.9515, 3381.6597, 3353.8961, 3364.0740, 3355.8001, 3447.1228, 3384.0178 $(M+H)^+$, respectively, consistent with the calculated molecular weight of designed peptides compared to that of crude material which exhibited multiple peaks along with the correct mass. RP-HPLC chromatogram as well as MALDI-tof mass spectrum of peptide VIII instantly was shown in Figures 1 and 2, respectively.

3.3. Amino acid analysis

The purified peptides were further characterized by amino acid analysis. As described in materials and methods section, the peptides after hydrolysis by 6 N HCl, were analyzed using cation exchange chromatography with post column derivatization. The results of ion exchange chromatography for hydrolyzed and derivative peptide VIII for instance was shown in Tables 2 and Figures 3 and 4. The areas under the curves for each peak were divided by those of standard (norleucine) and the amount of the residues in each sample was calculated according to the calibration equations of each residue. Finally the quantity of each residue in the peptide molecules was calculated. According to the observed residue quantity of the peptide and comparison of them with the calculated quantity shown in bar graphs of Figure 4, the structures of the peptides were in consistent with the expected structure.

3.4. Effect of the designed peptides against furin activity

In order to examine *in vitro* furin inhibition by designed peptides, a substrate namely IQVEGF-C (intramolecularly quenched

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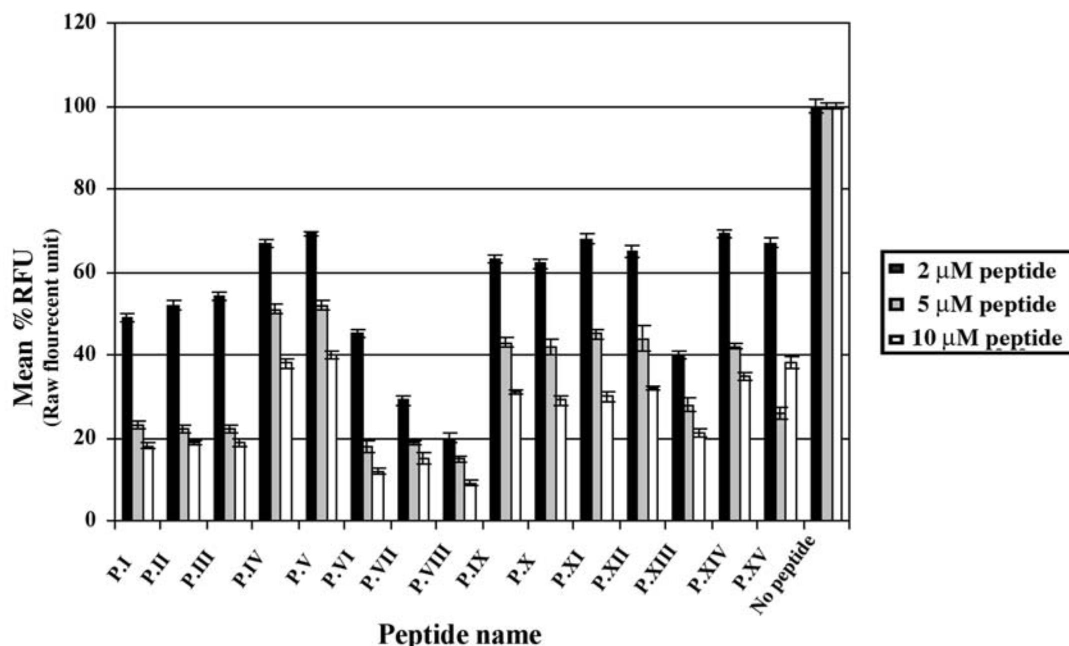

Figure 7. Bar graphs based on stop time assay to evaluate the effect of the designed peptides against furin activity after 60 min. incubation of the digestion mixture, as measured with Q-VEGF-C (10 μM) as a substrate (n = 3).

vascular endothelial growth factor), [Abz-QVHSIIRR^{227-} SLP-Y (NO_2)-A-$CONH_2$] were selected.

Human VEGF-C, a potential marker of tumorigenesis, is a protein of 419aa residues with a predicted molecular mass of 59 kDa. It is intracellularly processed from a precursor named pro-VEGF-C, that is a proprotein consisting of an N-terminal signal sequence (aa 1-12) followed by an N-terminal propeptide (aa 13–102), the VEGF homology domain (aa 103–227), and a cysteine-rich C-terminal segment (aa 228–419). ProVEGF-C is secreted as a disulfide-bonded homodimer that is proteolytically processed to release active form of the peptide. Upon examination of the amino acid sequence of the VEGF-C precursor, a dibasic motif, 220Q-VHSIIRR ↓ SLP230, resembling those recognized by the PCs is found, suggesting the involvement of these convertases in the maturation of VEGF-C. In fact furin was later found to be the processing enzyme [16].

In order to examine *in vitro* the processing effects of furin on the maturation of pro-VEGF-C, an intramolecularly quenched fluorogenic (IQF) peptide, IQ-h-VEGF-C [Abz-220QVHSIIRR↓SLP230-Y(NO^2)-A-$CONH^2$] containing the processing site of proVEGF-C was synthesized and characterized *in vitro* previously by Khatib and coworkers using an automated solid-phase peptide synthesizer. Abz is 2-(*O*)-aminobenzoic acid (fluorogenic group) and Y(NO_2) is 3-nitrotyrosine (fluorescence quench group) [16]. According to the results of this study IQ-VEGF-C is a good substrate for furin in the case of *in vitro* studies and can be used to evaluate the effect of various parameters, like inhibitors, in furin activity [16].

By the cleavage after IIRR$^-$ site in this substrate, the highly fluorescent compound of 2-amino benzoic acid was released and monitored using spectrofluorometer instrument. The intramolecularly quenched fluorogenic (IQF) substrate contains the physiological furin activation site of human

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proVEGF-C [16].

Three different concentrations of the designed peptides (2, 5 and 10 μM) were prepared in dH_2O, and furin activity to cleave the above mentioned substrate was monitored in the absence and presence of the designed peptides, by both on line and stop time fluorescence spectroscopy.

The results of stop time assays using 2, 5 and 10 μM of peptides I to XV at 10, 30 and 60 min using IQ-VEGF-C as substrate, were shown in Figures 5 to 7, respectively. It is evident that the pattern of inhibition of furin by the peptides is time and concentration dependent as it can be observed from the comparison of the Figures 5 to 7, and the comparison of bar graphs related to different concentrations in each figure. In the case of peptides I, II, III, VI, VII, VIII and XIII in all of incubation times and concentrations, an inhibition is monitored and intensified by increasing the concentrations and incubation times. In spite, in the peptides IV, V, IX, X, XI, XII, XIV and XV almost there is no inhibition at the first 10 min. in any of concentrations; in fact slight activation is noted in some cases. However, after 30 and 60 min. of incubation, relative inhibition is considered and heightened by increasing the concentrations.

According to a study of Jean *et al.*, the inhibition of furin by α1-PDX obeyed slow-binding inhibition kinetics whereby rapid formation of a loose α1-PDX-furin complex is followed by a slow isomerization to the tightly bound product [21]. Therefore, in the case of peptides IV, XI, XII, XIV and XV, the presence of more Arg or Lys residues at the positions P2, P6 and P8 may convert the peptides to an attractive substrate for furin, as it was shown by Klimpel *et al.* [24] as well as Molloy *et al.* [25] that furin cleaves an Arg-Xaa-(Lys/Arg)-Arg site with an E10-fold higher efficiency than that of Arg-Xaa-Xaa-Arg [24, 25]. Also three research groups have used a series of fluorogenic peptides to screen their effectiveness as substrates for furin. These studies have revealed that the Arg residues at the P_4 and P_1 positions are absolutely required for cleavage by furin. A basic residue (Lys or Arg) at the P_2 position enhances the cleavage efficiency [21, 26, 27]. These basic residue requirements are in good agreement with those determined by *in vitro* studies using protein substrates [24, 27, 28].

Thus, before the loose complex of furin-peptide could invert to a tightly bound product, the designed peptides were cleaved and degraded by furin. On the other hand, replacement of Arg or Lys by His at the positions P_2, P_4 and P_6, as well as mutating IL to Lue or Val at the position P_3 intensified the inhibitory activity of the designed peptides. It can be concluded that the presence of straight rather than branch alkyl chain containing amino acid at P_3 position can be stabilized the peptide-furin complex and lead to a more potent inhibition.

Acknowledgements

The authors gratefully acknowledge the financial support from Ministry of Health and Medical Education, government of Iran for the award of scholarship to Farzaneh Lotfipour. The funding from CFI, government. of Canada and OIT, Govt. of Ontario and CIHR is also gratefully acknowledged.

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