Nonhistone protein purified from porcine kidney acts as a suicide substrate inhibitor on furin-like enzyme[1]

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KEY WORDS proprotein convertase；furin；non-histone chromosomal proteins；enzyme inhibitors

ABSTRACT

AIM：To search and purify a naturally occurring protein inhibitor of the furin-like enzyme from the porcine kidney. METHODS：Recombinant kexin，a furin-like enzyme，from the yeast secretion expression was used as a target enzyme. The inhibitor component was extracted and purified from the acetone powder of porcine kidney. The inhibitory activity was monitored using a fluorogenic peptide substrate Boc-Arg-Val-Arg-Arg-MCA at spectrofluorimeter. RESULTS：The purified inhibitor component is a basic protein with an isoelectric point over 9.5. Its partial N-terminal sequence of 22 residues was determined，showing a high homology with nonhistone chromosomal protein HMG-17 in which there are four sites composed of dibasic residues，susceptible to be cleaved by the furin-like enzyme. This nonhistone protein could strongly compete with the fluorogenic substrate. However，this nonhistone protein would be degraded as a substrate by kexin if it was incubated with the enzyme for long time before adding the fluorogenic substrate，and subsequently lost its temporary inhibitory activity. CONCLUSION：The nonhistone protein isolated from the porcine kidney functioned as a suicide substrate inhibitor for the furin-like enzyme.

INTRODUCTION

The processing of precursor proteins via limited proteolysis at paired basic amino acids is an important and widely used cellular mechanism for the generation of biologically active proteins and peptides. Proprotein convertases （PC） are mainly involved in this endoproteolytic processing. The only unequivocal example of this endoprotease was the product of the kexin gene of the yeast *Saccharomyces cerevisiae*[1]. Its mammalian counterparts were identified in mouse pituitary and human insulinoma called PC1 and PC2，respectively[2,3]. Among proprotein convertases，furin localized in the trans-Golgi network is more popular as it is present in all tissues and cell lines examined[4]. Because of the homology of their catalytic domains to that of the bacterial serine protease，subtilisin，these enzymes are also called subtilisin-like proprotein convertases （SPC）[5].

The important substrates for proprotein convertase include not only most peptide hormones and neuropeptides，but also many growth factors，receptors，adhesion molecules，serum proteins，plasma proteases，and matrix metalloproteinases[6,7]. In addition to endogenous proproteins，many pathogens of bacterial exotoxins and viral-envelope glycoproteins also require these enzymes to be activated such as diphtheria toxin[8]，Shiga toxin[9]，Anthrax toxin PA[10] or to generate infectious virions such as Ebola virus glycoprotein[11]，HIV type gp160[12]，Newcastle-disease virus Fo[13].

Attempts have been made to develop protein-based furin inhibitors，since tissue- or cell-type-specific expression of these inhibitors controlled by a characterized promoter could be therapeutically valuable. A variant of α_1-antitrypsin （α_1-PDX） has been bioengineered to be an inhibitor highly selective for furin，and has the potential to be applied as an antipathogenic agent[14]. A widely expressed ovalbumin-type serpin，human proteinase inhibitor 8 （PI8），was also demonstrated to be an inhibitor of furin *in vitro*[15]. Besides，histidine-rich human salivary peptides are found to be moderately potent inhibitors for the furin-mediated cleavage of the fluorogenic peptide substrate pGlu-Arg-Thr-lys-Arg-MCA[16].

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In the present paper, we described that the purified nonhistone protein from the porcine kidney appeared to be a potent inhibitor of kexin though temporarily. Thus, this nonhistone protein can be regarded as a substrate suicide inhibitor of the furin-like enzyme, a similar mechanism of action could be found in α_1-PDX[14].

MATERIALS AND METHODS

Materials Fluorogenic substrate Boc-RVRR-MCA was purchased from Bachem, DEAE-cellulose, CM-cellulose from Whatman, QAE Sephadex A-25, Hitrap Mono S from Pharmacia, Ultrasphere C18 column from Beckman. Host cell, *S cerevisiae* strain CB018, and expression vector pG5-prokexin were gifts given by Dr RS Fuller of the University of California, Berkeley. 510 HPLC pumps and 996 photodiode array detector were from Waters-Millipore. Fluorescent spectrometer was manufactured by Hitachi.

Expression of recombinant kexin The transformed yeast strain CB018 with plasmid pG5-prokexin was grown in YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose, 50 mmol·L^{-1} Tris-HCl, pH 7.2) at 30 ℃ for 2 – 3 d. After centrifugation at 5000 × *g* for 5 min, the supernatant was collected, and stored at – 70 ℃.

Enzymatic assay The enzyme activity of kexin was monitored at 37 ℃ in a final volume of 1 mL containing fluorogenic MCA substrate 2 μmol·L^{-1} in Hepes buffer 20 mmol·L^{-1}, pH 7.5, $CaCl_2$ 0.1 mmol·L^{-1}, 0.05 % triton X-100 and β-mercaptoethanol 0.1 mmol·L^{-1}. For each assay, an equivalent amount of enzyme was added to release 12.5 nmol · L^{-1} · min^{-1} amino-4-methylcoumarin in the period of 5 min of enzyme reaction. The fluorescence of the released amino-4-methylcoumarin was measured on-line with a Hitachi spectrofluorimeter using an excitation and an emission wavelength of 380 nm (slit width, 10 nm) and 460 nm (slit width, 10 nm), respectively. For the determination of the inhibitor activity, a fixed amount of kexin solution was mixed in turn with different amounts of an inhibitor solution. After 5-min incubation the residual enzyme activity of each mixture was then determined. With increasing concentration of the inhibitor an inhibition curve could be plotted for estimation of the enzyme-inhibitor dissociation constant.

Preparation of porcine kidney acetone powder One kg of the fresh porcine kidney was cut into small pieces and stored at – 20 ℃. The frozen porcine kidney was homogenized in a Waring blender with 5 times volumes of cold acetone (– 20 ℃). The tissue residue was squeezed thoroughly with a piece of cloth, and further treated with cold acetone two or three times in order to remove aqueous solution from the residue as completely as possible. The residue was then dried at room temperature and around 130 g acetone powder could be obtained.

Purification of the inhibitor component Fifty gram of the acetone powder was homogenized in 400 mL of 2.5 % (w/v) trichloroacetic acid (TCA) in a Waring blender, and centrifuged at 4500 × *g* for 15 min. As the inhibitor component was adhered to the bulk of precipitate, the pellet was washed twice in 300 mL of 2.5 % (w/v) TCA. All supernatants were then combined and stored at 4 ℃. After adjustment of pH and ion strength, the solution extracted from the acetone powder was loaded on a cation-exchange column (CM-cellulose 52, 2.5 × 8 cm) equilibrated with HAc-NaAc buffer 70 mmol/L, pH 4.3. A stepwise elution was performed with NaCl 0.25 and 1.0 mol/L in the same buffer at a flow rate of 2 mL/min. Most of the inhibitory activity was found in the fraction of 1.0 mol/L NaCl elution, which was concentrated and desalted on a column of Sephadex G-10. The sample was then applied to an anion-exchange column (QAE-Sephadex A-25, 1.5 × 10 cm) equilibrated with Glycine-NaOH 100 mmol/L, pH 9.5, buffer at a flow rate of 1 mL/min. The breakthrough fraction was pooled, its pH value and ion strength were adjusted, and loaded on a small column of Mono S HR5/5 equilibrated with HAc-NaAc buffer 100 mmol/L, pH 4.0, on the 510 HPLC instrument. The column was eluted at flow rate of 2 mL/min with a linear gradient of NaCl 0 – 1.0 mol/L in the elution buffer for 50 min. Fractions with an inhibitory activity were concentrated, desalted, and rechromatographed. The sample was then applied on a reverse-phase HPLC column (Beckman Ultrasphere C18) equilibrated with 0.1 % TFA, and eluted with a linear gradient of 0 – 60 % acetonitrile in 0.1 % TFA at flow rate of 1 mL/min for 60 min. Purification procedures were carried out at room temperature (10 – 20 ℃). The purified inhibitor component was then obtained after second HPLC, and used for amino acid sequence determination.

Amino acid sequence determination The N-terminal partial amino acid sequence of the inhibitor component was determined on a Beckman model LF3200 peptide/protein sequencer and PTH-analyzer using the program provided by the manufacturer.

RESULTS

Purification of the inhibitor component The purification results of the inhibitor component in each step are summarized in Tab 1.

The component was purified over 3000 folds. As the component is a very positively charged protein, most of contaminants were removed by anion-exchange chromatography on the QAE-Sephadex column eluted with a high pH buffer. This step resulted in 90-fold purification with a good yield. The other contaminants were removed by HPLC on a small column of Mono S HR 5/5 (Fig 1), followed by reverse phase HPLC on a C18 column (Fig 2). Because the component is a strong basic protein, easily adhered to the negatively charged glassware leading to a decrease in yield, all glassware and plastics were siliconized.

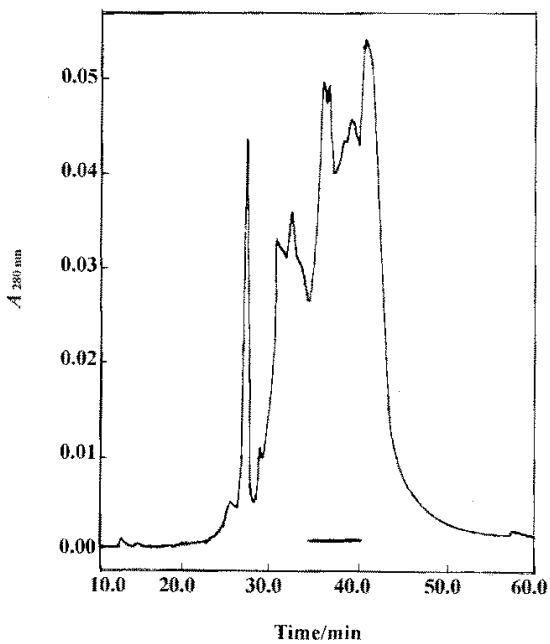

Fig 1. Chromatography of the inhibitor component on Mono S HR 5/5. Elution was performed at flow rate of 2 mL/min with a linear gradient of 0 – 1.0 mol/L NaCl in the elution buffer for 50 min. Solution A was 100 mmol/L HAc-NaAc, pH 4.0, and solution B 1.0 mol/L NaCl in the same buffer. The inhibitory activity was found in the fractions as indicated by a bar.

Inhibitory activity on kexin The purified inhibitor component displayed a potent inhibitory activity on kexin as shown in Fig 3. Under 50 % inhibition the dissociation constant of this component could be estimated to be around 3 nmol·L^{-1}, this value fell into the average range for most of protease inhibitors. However, this inhibitory activity decreased along with an extension of incubation time. For 30- and 90-min instead of 5-min incubation of the inhibitor component and the enzyme before the substrate was added, its inhibitory activity decreased to 50 % and 10 %, respectively. It meant that the component was degraded progressively by kexin during long time incubation. Thus the purified component would not be a real inhibitor of kexin, but could rather be regarded as a suicide substrate inhibitor.

Determination of the partial N-terminal sequence The partial N-terminal sequence of 22 residues of the purified inhibitor component was determined on a Beckman model LF3200 protein sequencer to be Pro-Lys-Arg-Lys-Ala-Glu-Gly-Asp-Thr-Lys-Glu-Asp-Lys-Pro-Lys-Val-Lys-Asp-Glu-Pro-Ser-Arg. The sequence was then submitted to Genbank using BLASTP 2.0.7 for a protein similarity searching. It was found that this partial N-terminal sequence is highly homologous to that of non-histone chromosomal protein HMG-17, sharing 82 % identity. Their different residues are boxed as shown in the next page.

Obviously the inhibitor component purified from the porcine kidney should belong to the nonhistone family. It is worth pointing out that in the molecule of nonhistone HMG-17 there are four pairs of dibasic residues located in the positions 3 – 4, 23 – 24, 42 – 43 and 56 – 57, all of them should be susceptible to the furin cleavage.

Tab 1. Purification of the inhibitor component from the porcine kidney.

Purification step	Total protein/ mg	Specific activity* / nmol·μg^{-1}·min^{-1}	Recovery/ %	Purification/ Fold
Extraction of the acetone powder	3820	0.98	100	1
CM-Cellulose	1266	2.08	70	2
QAE-sephadex A25	13.2	187.5	66	191
Mono S HR 5/5	0.35	3409	31.8	3478
Ultrasphere C18 HPLC	0.19	3810	19.3	3887

*The specific activity was expressed in the amount of AMC released by kexin inhibited by one μg of protein in the assay system.

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PKRKAEGDT KEDKPKVKDE ESR- (the inhibitor component from the porcine kidney)

MPKRKAEGDA KGDKAKVKDE EQRRSARLSA KPAPPKPEPK PKKAPAKKGE KVPKGKKGKA DAGKEGNNPA
10 20 30 40 50 60 70

ENGDAKTDQA QKAEGAGDAK (nonhistone chromosomal protein HMG-17)
80 90

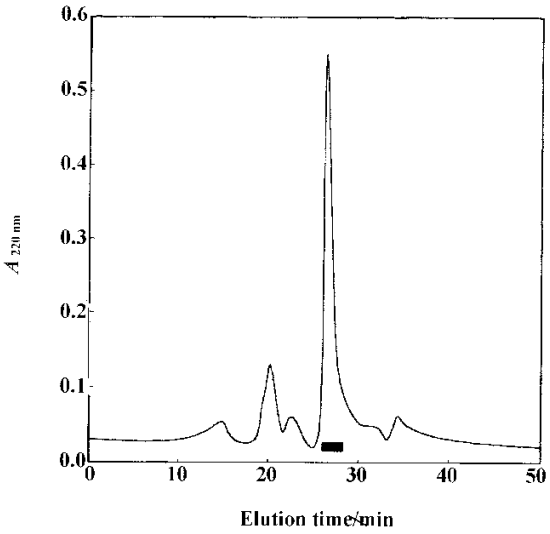

Fig 2. Chromatography of the active fractions from Mono S on Reverse-phase HPLC. The C18 column (4.6 × 250 mm) was eluted with the trifluoroacetic acid (TFA)-acetonitrile linear gradient system (0 – 50 % solution B) in 50 min. Eluting solution A was 0.1 % TFA in water; solution B was 0.1 % TFA, 70 % acetonitrile in water. The peak with the inhibitory activity was marked by a bar.

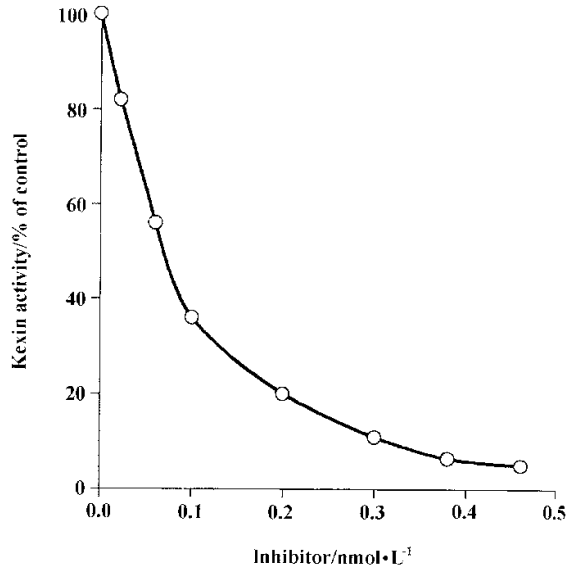

Fig 3. Inhibition curve of the nonhistone-like protein of the inhibitor component toward kexin. The molecule weight of this nonhistone-like protein was presumed to be about 10 kDa. The IC_{50} value of the protein is around 10 nmol/L.

DISCUSSION

The broad and important role of furin-like endoproteases in the proteolytic maturation of not only endogenous proprotein substrates, but also of activation of many exogenous pathogens such as viral envelope glycoproteins and bacterial toxins, has made these convertases as targets to search for their potent and selective inhibitors[6,7]. However, up to now there is no report on any naturally occurring inhibitor active on any proprotein convertase. A potent inhibitor to kexin, a furin-like enzyme, from the porcine kidney now has been found and purified. Its partial N-terminal sequence shares 82 % similarity with that of nonhistone chromosomal protein HMG-17. According to its cDNA deduced amino acid sequence, this nonhistone is a very positively charged protein composed of 90 residues including 26 basic residues. Among these there are four sites of paired basic amino acids, susceptible to be cleaved by the furin-like enzyme. As a result, the nonhistone-like protein purified from the porcine kidney is capable of strongly competing with the fluorogenic peptide substrate for kexin when the enzyme and the inhibitor are incubated for a short time. However, such inhibitor is not stable, and will be degraded by the target enzyme during a long incubation time. It can be fully understood if the structure of the nonhistone protein is taken into account. In this protein there is no cysteine residue for forming a disulfide bridge, no aromatic residue and lack of hydrophobic residues for forming a hydrophobic core inside of the protein molecule. Both of them are basic elements of the secondary structure of protein and form a stable and rigid molecule. Furthermore nonhistone proteins are rich in proline residues, there are up to 10 residues of proline in nonhistone HMG-17. As is well known the proline residue interfere with the formation of α-helix structure, which is also an important element for a steady structure of protein. Considering all these factors, the nonhistone-like protein purified from the porcine kidney could not be a real inhibitor to the furin-like enzyme, and could be regarded only as a suicide

substrate inhibitor. In fact, the variant of α_1-antitrypsin which also exerts an inhibitory activity on the furin-like enzyme is also no other than a suicide substrate inhibitor[14].

The nonhistone protein is considered to bind to the inner side of the nucleosomal DNA thus altering the interaction between the DNA and the histone octamer. Its function may be involved in the specific gene transcription[17]. Whether the additional inhibitory activity function of this protein plays some role in the regulation of proprotein convertase in physiological condition within the cells remains to be clarified.

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猪肾中纯化的非组蛋白是 furin 样酶的自杀性底物抑制剂[1]

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关键词 前体蛋白加工酶；furin；非组染色体蛋白质类；酶抑制剂

目的：从猪肾脏中寻找并纯化 furin 样酶的天然抑制剂. 方法：通过酵母分泌表达系统获得 furin 样酶的重组 Kexin. 从猪肾丙酮粉中抽提并纯化抑制剂组分. 抑制剂活力在荧光分光光度计上，用荧光底物 Boc-Arg-Val-Arg-Arg-MCA 测定. 结果：纯化到的抑制剂组分是一等电点超过 9.5 的碱性蛋白. 测定了其 N 末端 22 个残基的序列. 该序列与非组蛋白 HMG-17 高度同源，后者含有 4 个易被 furin 样酶裂解的双碱性氨基酸位点. 因此，该非组蛋白可与荧光底物强烈竞争. 若将酶与非组蛋白长时间温育，其抑制剂活力将最终丧失. 结论：猪肾中纯化的非组蛋白是 furin 样酶的自杀性底物抑制剂.

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