链脲佐菌素致胰岛 NO 自由基损伤模型的建立和应用

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摘 要 以链脲佐菌素 Streptozotocin(简称 STZ)为糖尿病的诱因,以 NO 自由基含量为响应指标,建立了体外小鼠胰岛水平糖尿病药物筛选模型。当 STZ 作用浓度在 0 ~ 5.0mmol/L 内变化时,培养液中被检测到的 NO 大部分是来源于 STZ 溶于水后释放出的,而很小一部分是由胰岛培养物自身释放的,后者稳定在 30 ~ 35mmol/L 之间。另一方面,NO 含量与胰岛素分泌量的剂量关系表明 NO 的增加伴随着胰岛素分泌量的下降,这标志着 NO 对胰岛功能的氧化性损伤,从而验证了 NO 作为该模型响应参量的可靠性。最终确定 STZ 致胰岛 NO 自由基损伤模型中 STZ 的作用浓度为 5.0mmol/L,此时 NO 含量和胰岛素分泌量分别为 STZ 未加入前的 10.81 倍和 0.43 倍。最后应用该模型,快捷地考察了不同铬含量的魔芋葡甘露寡糖铬络合物(简称 KOSCr)清除 NO 自由基的能力。

关键词 链脲佐菌素, NO, 自由基, 模型
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糖尿病(Diabetes mellitus)是目前世界上最大的公众健康问题之一,是由于体内胰岛素分泌的绝对或相对不足而引起的人体糖、脂代谢的紊乱[1]。氧自由基在阿脲、链脲佐菌素 Streptozotocin(简称 STZ)和自身免疫性糖尿病的发病过程中以及在 β 细胞损伤和影响胰岛素分泌方面都是重要因素[2]。其中 NO 自由基可能作为 β 细胞损伤的中介者[3]。

建立药物筛选模型是实现高通量药物筛选的核心[4],相对于整体动物模型,细胞和分子水平模型具有特异性高、成本低、适合高通量筛选、操作简便等优点[5,6]。针对糖尿病药物筛选,目前已建立了成熟的动物模型[7,8],但细胞水平筛选模型的研究还未广泛开展。同时,保持完整的胰岛组织结构是各类胰岛细胞行使和维持正常生理功能所必需的[9]。

本论文工作将在体外胰岛水平建立 STZ 致 NO 自由基损伤糖尿病药物筛选模型,并应用该模型,初步探讨魔芋葡甘露寡糖铬络合物(简称 KOSCr)清除 NO 自由基的能力。

1 材料与方法

1.1 材料与仪器

柠檬酸、萘乙二胺盐酸盐(NEDD)、磺胺、$CrCl_3 \cdot 6H_2O$,柠檬酸三钠均为分析纯,购自北京化学试剂公司;无糖 RPMI-1640 培养基购自 Gibco BRL 公司;胰岛素放免检测试剂盒,购于中国原子能科学研究院;γ-计数仪,型号为 FT-630G,北京核仪器厂生产;三周龄 ICR 小鼠,北京大学医学院实验动物中心提供;胎牛血清、青霉素、硫酸链霉素、Hepes、胶原酶Ⅳ型、透明质酸酶、DNase Ⅰ、STZ,均购自华美生物工程公司;魔芋葡甘露寡糖铬络合物(简称 KOSCr)为实验室自己合成,原料魔芋精粉由云南昭通制药厂提供,β-甘露聚糖酶由云南大学提供。

1.2 方法

1.2.1 STZ 液配制[10]:以中性 PBS 配制 0.1mmol/L 柠檬酸,并用 3mmol/L NaOH 调节 pH 至 5.0;以之为溶剂配制 0.1mmol/L 的 STZ 母液并经 0.22μm 膜过滤。用时用相应的缓冲液稀释。

1.2.2 NO 自由基含量检测[11]:用去离子水配制 0.1% NEDD,用 5%(*W*/*W*)磷酸配制 1%(*W*/*W*)磺胺液,将二者等量混合,抽真空得到 pH 为 2.4 的酸性 Greiss 试剂。待测样品与 Greiss 试剂等量混合,室温反应 10min,在 540nm 处测吸光值。

1.2.3 放射免疫分析法(简称 RIA)检测胰岛素含量[12]:以浓度为 5 ~ 160μIU/mL 的胰岛素标准品绘制

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标准曲线。待测样按照试剂盒中操作步骤反应后，于 γ-计数仪测定沉淀读数，即可得到相应胰岛素含量。

1.2.4 体外胰岛组织分离及原代培养 :胰岛收获的基本方法参照 Ramanadham 的方法[13]。分离到的胰岛以 RPMI-1640 为培养基，37℃，5% CO_2 培养箱中培养。

1.2.5 实验数据处理 :实验数据为 3 次平行实验检测结果的平均值，表达为平均值 ± 标准误差的形式。

1.2.6 KOSCr 中铬含量测定 :日立 18050 型原子吸收分光光度计，测定波长 357.9nm，电流 7.5mA，狭缝 1.3nm，空气压力 1.6kg/cm^2，乙炔气压力 0.35 kg/cm^2。

2 结果与分析

2.1 体外小鼠胰岛分离与原代培养

本工作对消化所采用酶的种类、用量及消化时间进行了优化。实验中 0.1%（*W*/*V*）胶原酶Ⅳ型、0.1%（*W*/*V*）透明质酸酶和 0.1%（*W*/*V*）DNase Ⅰ的用量分别为组织碎片体积的 4、2 和 1 倍。其中 DNase Ⅰ的引入可剔除消化过程中因部分细胞破损而泄露的遗传物质。由于胰岛是分散排布在胰腺内分泌部的，实验中在 10min、37℃热消化过程之前先进行了 2h、4℃的冷消化处理，该过程有利于酶分子扩散进入组织块内部，以实现组织块内外的同步消化。结果表明，该方法可使活性胰岛的收获率提高 10%，胰岛原代培养物可在体外存活 6 周，维持稳定分泌功能 10d，能适应糖尿病药物筛选的初步要求。

2.2 验证模型中 NO 的来源

STZ 溶于水会释放出大量 NO，胰岛受到 NO 的损伤性攻击后自身也可能产生部分 NO。为了验证模型中 NO 的主要来源，分别在有与没有胰岛时，采用 0 ~ 5.0mmol/L STZ 溶液，其他实验条件相同时做 NO 检测。结果如表 1 所示，由胰岛组织自身释放的 NO 不会随 STZ 浓度而发生显著性变化，维持在 30 ~ 35mmol/L 内，而培养环境中的 NO 随 STZ 浓度的增加而增加，可以判断出主要是由于 STZ 溶于水后产生的。所以模型中 NO 的含量主要与 STZ 的浓度有关。

2.3 验证 NO 自由基作为模型的主要性能指标

实验中采用 0 ~ 5.0mmol/L STZ 刺激胰岛，检测相应胰岛素水平的变化，以明确 STZ 浓度与胰岛分泌功能的量效关系，并考察培养环境中 NO 含量与胰岛素分泌量的剂量关系。

表 1 有和没有胰岛存在时不同 STZ 作用浓度下的 NO 含量

Table 1 NO level under different STZ concentrations in the absence or presence of islets

	0mmol/L STZ	1.0mmol/L STZ	5.0mmol/L STZ
NO level without islets(mmol/L)	0	73.75 ± 17.41	378.47 ± 15.26
NO level with islets(mmol/L)	31.82 ± 0.95	108.48 ± 4.91	411.05 ± 7.56
Average NO release from islets(mmol/L)	31.82	34.73	32.58

2.3.1 数据处理方法 :以含有一定浓度 STZ 的 KRB 缓冲液培养胰岛 2h 后，收集上清检测 NO 含量。考虑到各孔板中实际接种的胰岛数目会由于胰岛个体形态的差异而存在不同，所以 NO 含量采用相对值（标记为 Y/X）来表示，其中基准值（Basal）X 为以含有 11.1mmol/L 葡萄糖的 KRB 液培养胰岛 2h 后上清液中的 NO 含量，Y 为在实验条件下培养 2h 后的 NO 含量。胰岛素分泌量的计算方法同上，也以相对值来表示。

2.3.2 结果 :在 0.5 ~ 5.0mmol/L 的 STZ 作用下，NO 含量和胰岛素分泌水平如图 1 所示。

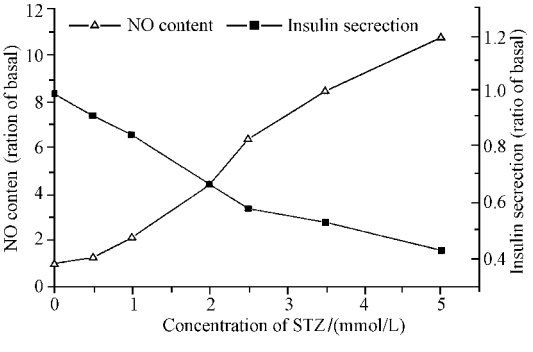

图 1 STZ 作用于胰岛时 NO 自由基含量与胰岛素分泌量的剂量关系

Fig.1 Dose-dependent relationship of NO content and insulin secretion of STZ treated islets

环境中 NO 含量随 STZ 作用浓度的增加呈现出明显的正相关性增加，而胰岛素分泌水平呈现反相关性下降。结合表 1 结果可以推断，STZ 溶于水后释放出大量的 NO 自由基，后者攻击胰岛会造成胰岛分泌功能的障碍或损伤，表现为胰岛素分泌水平的降低。最终确定，5.0mmol/L STZ 即可成功地制造体外胰岛糖尿病模型，此时 NO 含量和胰岛素分泌量分别为 STZ 加入前的 10.81 倍和 0.43 倍。由于模型中胰岛功能的损伤主要与 STZ 释放出的大量 NO 自由基相关，所以凡是能清除或抑制自由基的化

合物在该模型中应表现为可一定程度上降低 NO 的含量,从而保护胰岛免受自由基侵害,这也正是建立此胰岛水平糖尿病药物筛选模型的目的。

2.4　不同铬含量对 KOSCr 清除 NO 自由基效果的影响

初步探索发现 KOSCr 较 KOS 本身有较好的 NO 清除效果。为进一步考察过渡金属离子 Cr^{3+} 的加入对 KOS 生物活性的影响,实验中利用 STZ 致胰岛 NO 自由基损伤模型测定了一系列不同铬含量的 KOSCr 清除 NO 自由基的效果。结果如图 2 所示。

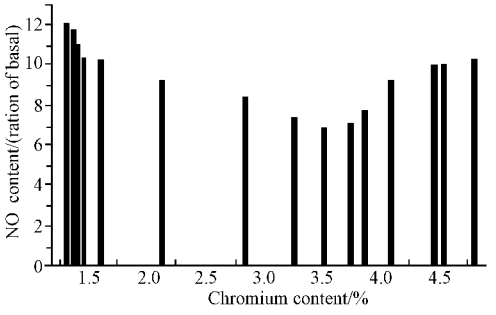

图 2　铬含量与 NO 含量

Fig.2　The relationship of chromium content and NO content

实验中设定的模型对照组(5.0mmol/L STZ)和 KOS 对照组(1mg/mLKOS,5.0mmol/L STZ)中环境 NO 含量测定值分别为 10.81 和 9.008,可以看出 KOS 本身就有较好的清除 NO 的效果。KOSCr 铬含量在 2.842% ~ 3.871% 之间时无论与模型对照组还是 KOS 对照组相比其降低 NO 释放量的效果都很显著,从而起到保护胰岛的作用,其中铬含量增加到 3.519%时效果最显著,而超过该值后,效果却逐渐减弱。初步推测由于络合物中铬离子与 KOS 中羟基中的孤对电子发生配位结合,引起 KOS 空间构象的改变,从而在后者参与的细胞识别过程中起到一定作用。在实验设定的络合物对照组中发现,单纯以络合物与胰岛共培育 2h, NO 含量为 1.244,与空白对照组(仅 KRB 缓冲液)持平,表明 KOSCr 本身对正常胰岛无不良影响。

可见,通过 STZ 致胰岛 NO 自由基损伤模型可以快速方便地进行络合物清除 NO 自由基活性的筛选,为其今后产品的质量和稳定性研究提供了理论依据。

3　结　论

本文以体外小鼠胰岛原代培养为操作平台,建立了 STZ 致胰岛 NO 自由基损伤糖尿病药物筛选模型。与动物模型相比,该模型具有操作方便简单、检测准确、成本低的优点,并能使胰岛保持完整的结构而行使其胰岛素分泌功能。利用该模型可加快糖尿病药物筛选的速度和准确度。但经过该模型筛选得到的有活性的化合物仍需做进一步的动物实验以进行活性确认。有关魔芋葡甘露寡糖铬络合物清除 NO 自由基的能力以及铬含量与活性之间的关系仍需做进一步的研究与探讨。

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Establishment and Application of the Model of Islet Impaired by NO Free Radical Released from Streptozotocin

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Abstract For diabetes mellitus, little research has been done on the tissue-based or cell-based drug screening model, which has advantages over traditional animal diabetic model in high specificity, high screening volume, low cost and simple manipulation. Considering that the maintenance of complete islet tissue structure is the prerequisite for islet cells to perform their functions normally, an *in vitro* islet-based drug screening model for diabetes mellitus was established and evaluated.

Pancreatic islets were isolated from 3 weeks old mice of either sex by collagenase digestion and density gradient centrifugation as prescribed by Ramanadham S. The volume of 0.1% (*W*/*V*) collagenase Ⅳ, 0.1% (*W*/*V*) Hyaluroridase and 0.1% (*W*/*V*) DNase Ⅰ were 4 times, 2 times and 1 times that of the islets to be digested. And a 2 hours' cold digestion at 4℃ was followed by a 10 minutes' warm digestion at 37℃. Under the optimized digestion condition, the islet recovery could be increased by 10%. The isolated islets could survive 6 weeks in vitro and show stable insulin secretion in the first 10 days after inoculation. The obtained islets were cultured in RPMI-1640 medium at 37℃ with 5% CO_2.

Then a diabetic model was established by selecting streptozotocin (STZ) as the evocator and nitric oxide (NO) as the responding index. After 1 day's inoculation, islets culture was treated with STZ, whose concentration ranged from 0 to 5.0 mmol/L. NO was measured by a colorimetric assay at 540nm based on the Griess reaction for 10min with 0.1mL Griess reagent and 0.1mL culture supernatants. Insulin secretion was assayed by RIA methods. Due to the islets-related inoculation variations, NO release and insulin content were both expressed as a percentage of the value recorded in basal experiment which was in the only presence of Krebs culture medium. It was testified that the amount of NO released from islet itself remained steady at 30 ~ 35mmol/L regardless of the changes of STZ concentration from 0 to 5.0mmol/L. However the NO content in the supernatants of islets culture had close relationship with STZ concentration. This indicated that in this STZ-induced islet diabetic model, NO mainly comes from STZ when it dissolves in water. On the other hand, when STZ changed from 0 to 5.0mmol/L, the dose-dependent relationship between NO content and insulin secretion showed that the increase of NO came along with the decrease of insulin secretion, which is an important symbol of islet function. As a kind of oxidative free radical, NO is capable of impair islet cells. Thus, NO is a reliable responding index of the model.

The optimal STZ concentration in the model is finally determined to be 5.0mmol/L, under which condition the NO content and insulin secretion is 10.81 times and 0.43 times that in the medium before STZ is added. So if anything is effective in lowering the NO content in the culture, it could protect islets cells from the oxidative attacks of NO.

Finally, as an application of the model, the scavenging effect of KOSCr on NO was studied. In a series of KOSCr with different chromium content, all had shown better NO scavenging effects than KOS itself, which could give us an enlightenment of the influence of chromium ion on oligosaccharide. And 1mg/mL KOSCr with 3.519% chromium content can significantly inhibit the NO formation. This has lain a theoretic basis for the research of KOSCr bioactivity and quality control. These results suggested that the STZ-induced diabetic islet model which is impaired by NO free radical can be used effectively, fast and conveniently when screening potential diabetes drugs.

Key words Streptozotocin, NO, free radical, model

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Molecular Cloning and Sequence Analysis of Ussurin, a New Metalloproteinases/disintegrin from *Gloydius ussuriensis*

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Abstract The metalloproteinases/disintegrins in the snake venom act as platelet aggregation inhibitor by an antagonism against integrin on platelet through its RGD sequence and may play other important role in cell-cell fusion, cell matrix interaction and other cellular function. Ussurin is a new metalloproteinase/disintegrin that was cloned from *Gloydius ussuriensis*. Poly (A$^+$) RNA was purified from the total RNA preparation from venom gland of a single *G. ussuriensis* using the poly (A$^+$) tract-mRNA isolation system. A cDNA library was constructed with the SMART PCR cDNA library construction kit. The cDNA library was screened and the positive clones were selected. The full-length cDNA of Ussurin was obtained. The cDNA encoding the Ussurin precursor has a 51bp 5′-UTR, the open reading frame of Ussurin and a 490 bp 3′-UTR, the open reading frame of Ussurin cDNA nucleotide sequence is 1434bp and codes for 478 amino acids with a predicted molecular mass of 53.2kD and an isoelectric point of 5.37. There is no potential N-glycosylation site in the deduced sequence region. Its deduced amino acid sequence consists of four region, a signal sequence of 18 amino acid residues, a zymogen pro-peptide of 171 amino acid residues with a cysteine switch motif (PKMCGVT) in it, a central metalloproteinase domain of 201 amino acid residues containing a conserved zinc-chelating sequence (HEXXHXXGXXH) and a methionine-turn CIM involving zinc banding also, a space sequence between metalloproteinase domain and disintegrin domain of 15 amino acid residues with a conserved T_{392}, T_{397}, S_{400}, which is specific residues of the P-Ⅱ snake venom metalloproteinases, a disintegrin domain of 73 amino acid residues with a characteristic RGD region and six-disulfide bonds. Ussurin belongs to P-Ⅱ class. The cDNA sequence and deduced amino acid sequence of Ussurin precursor were compared with homologous sequence in the GenBank database, the result reveals high degree of homology in sequence and organization pattern of domain with metalloproteinase/disintegrin gene family of other snake species. Compared with the alignment of amino acid sequence of metalloproteinase/disintegrin member, hypervariable regions of this member were revealed, besides they share higher homologous in the zymogen domain. It suggests that the hypervariable regions are the counterparts directly suitable for interacting with different domain of receptors, different receptors or substrates.

Key words metalloproteinase/disintegrin, molecular cloning, *Gloydius ussuriensis*, cDNA

In China, *Gloydius ussuriensis* is a kind of snake with great medical use. Snake venom metalloproteinases (SVMPS) are synthesized in venom gland as pre-pro-metalloprotease that are rapidly processed to the mature and functional proteinase. SVMPS belong to a reprolysin subfamily of the M12 metalloproteinases that contain a conserved Zinc-chelating sequence (HEXXHXXGXXH)[1-3]. The SVMPS have been divided into four groups (P-Ⅰ to P-Ⅳ) according to their size and additional domains organization following the metalloproteinase domain[2]. The P-Ⅰ class enzyme, containing the smallest member, is synthesized with only pre-pro-proteinase domain; The P-Ⅱ class of venom reprolysin contains a metalloproteinase domain, a short spacing peptide and a disintegrin domain after the carboxyl terminal. The P-Ⅲ SVMPS have a cysteine-rich domain carboxyl to the disintegrin-like domain of P-Ⅱ. The P-Ⅳ class has all of the domains of P-Ⅲ class and lectin-like domain at the carboxyl terminal. The disintegrins are peptides, derived from P-Ⅱ SVMP disintegrin domains and isolated from the venom of the crotalid and viperid snakes, range from 49 to 83 amino acid residues. Almost all disintegrins have the cell-

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The sequence data of Ussurin in this paper have been submitted to the GenBank database under the accession number AY204245.
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binding consensus RGD sequence (Arg-Gly-Asp) as characteristic structure. The RGD sequence locating in a flexible hairpin loop of 13-amino acid residues plays a crucial role in inhibition of platelet aggregation by interaction with platelet integrin $\alpha_{II}\beta_3$ [4,5]. Sequence analysis of both cDNA of hemorrhagins and disintegrins has shown that they are derived from the same metalloproteinase/ disintegrin gene family. Disintegrins are synthesized as large precursor molecules containing the Zinc-dependent proteinase domain found in hemorrhagic toxins, which the disintegrin domain is present in large metalloproteinases (50 ~ 70kD), and it is absent in low-molecular-mass hemorrhagins (20 ~ 25kD) [2, 6-9] In this report, we present the cDNA sequence of Ussurin, a metalloproteinases/disintegrin from *G. ussuriensis*.

1 Materials and Methods

1.1 Venom gland

The venom glands for cDNA construction of a single specimen of *G. ussuriensis* were obtained and immediately frozen in liquid nitrogen.

1.2 Poly(A) RNA Isolation

The glands were removed and placed into TRIzol Reagent (Gibco) and homogenized. Homogenate was laid on 5.7mol/L cesium chloride and centrifuged for 20h at 35 000r/min at room temperature. The precipitate RNA was recovered and precipitated in ethanol. Poly (A^+) RNA was purified from the total RNA preparation using the poly (A^+) tract-mRNA isolation system (Promega).

1.3 cDNA library construction

DNA synthesis was accomplished using the SMART PCR cDNA library construction kit (Clontech). First-strand synthesis from 1.0μg poly(A^+)RNA was primed by SMART nucleiotide sequence (TACGGCTGCGAGAAGACGACAGAAGGG and CDS/3′ PCR primer sequence(5′-ACGTGCGGCCGCGGATCC(dT)30) and catalyzed by superscript Ⅱ. The cDNA was amplified by long distance PCR by using primer above and advantage KlenTaqe Polymerase mix. The cDNA was digested with *Sfi* Ⅰ to produce the sticky end containing the *Sfi* Ⅰ linker/primer. The resultant cDNA had the *sfi* Ⅰ site at the both end.

After size-selection to expulse the large and the small segment, cDNA was then ligated into PDBS, a derivative vector of Strategene's pBluescript-sk which was made a *Sfi* Ⅰ site in multicloning site. The recombinant DNA was transformed into *E. coli* DH5α. The titer was found to be 2.5×10^7 recombinants.

1.4 Probe design

According to the high conservation of the predomain at the N-terminal, a 22 meric oligonucleotide probe was synthesized. Another adjustment probe of 20 mer was synthesized according to the conservative sequence of disintegrin at the 3′-terminal.

1.5 cDNA isolation

Clones of Ussurin *E. coli* DH5α containing recombinant cDNA were plated onto luris broth/ampicillin plates. Colonies resistant to ampicillin were transfered on to two sets of membrane copies and kept for colony hybridization with ^{32}P-end-labeled primers. Pre-hybridization was accomplished at 65℃ 4h in hybridization buffer. Each set of membranes was hybridized at 46℃ for 10h in 40mL of hybridization buffer containing 5pmol/L of one of two labeled oligonucleotide probes. Hybridized membranes were washed with 6 × SSC and 0.1% (*W*/*V*) SDS in turns of two times at 4℃ for 6 min, one time at room temperature for 6 min and once at 50℃ for 3 min. Clones pretended positive results in both set of membranes were further purified into single cell clone by comparing two sets of primers autoradiography.

1.6 DNA sequencing

DNA sequencing was performed on a model 3700 automated DNA sequencer, and the Taq Dye Deoxy terminator cycle sequencing kit was used. Sequence overlaps were obtained by using oligonucleotide primers to the internal sequence. Both strands were sequenced entirely. The cDNA sequence and deduced amino acid sequence were compared to sequences in the GenBank.

2 Result

The cDNA library made from the mRNA of *G. ussuriensis* venom gland was screened, because the metalloproteinases/disintegrin Ussurin is a highly expressed enzyme in the venom gland, the average number of clones that tested positive to both probe from the screening of five plates reached to 1.2%. Twenty clones were chosen from the positive for further work and five colonies were sequenced. Figure 1 shows Ussusin gene and its deduced amino acid sequence of the open reading frame. The open reading frame of Ussurin cDNA nucleotide sequence is 1434bp and codes for 478 amino acids with a predicted molecular mass of 53.2kD and an isoelectric point of 5.37. There is no potential N-glycosylation site in the deduced sequence region. Its deduced amino acid sequence consists of four regions, a signal sequence, a central metalloproteinase domain, a space sequence between metalloproteinase domain and disintegrin domain, a disintegrin domain. At the N-terminal, there was a typical signal 18-peptide with Gly^{18}-Ser^{19} motif that can be cut by a single peptidase.

There was a zymogen domain that was composed of 19-189 amino acids residues after signal peptide. A sequence of P^{164}-K^{165}-M^{166}-C^{167}-G^{168}-V^{169} was stringently conserved among snake venom proteinase and was cysteine switch motif that involved in the activation of metalloproteinase activation. The sequence of 190-390 amino acid residues was the metalloproteinase domain, which was followed by a spacer sequence of 391-405 amino acid residues, and a disintegrin-like domain of 406-478 amino acid residues. Six of seven cysteins involve the formation of 3 disulfide bonds of Cys^{304}-Cys^{384}, Cys^{344}-Cys^{351} and Cys^{346}-Cys^{368}, which seldom present in SVMPS. The unbounded cystein 373 is important to the proteinase catalysis activity.

In metalloproteinase domain, a significant amino acid sequence was H-E-X-X-H-X-X-G-X-X-G which was the zinc binding sequence previously described by Gomis-Ruth [8].

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1    ATG ATC CAG GTT CTC TTG GTG ACT ATA TGC TTA GCA GCT TTT CCT
1    M   I   Q   V   L   L   V   T   I   C   L   A   A   F   P
                   Signal peptide
46   TAT CAA GGG AGC TCT ATA ATC CTG GAA TCT GGG AAC GTG AAT GAT
16   Y   Q   G   S   S   I   I   L   E   S   G   N   V   N   D

91   TAT GAA GTA GTC TAT CCA CGA AAA GTC ACT GCA TTG CCC AAA GGA
31   Y   E   V   V   Y   P   R   K   V   T   A   L   P   K   E

136  GCA GTT CAG CCA AAG TAT GAA GAC ACC ATG CAA TAT GAA TTG AAA
46   A   V   Q   P   K   Y   E   D   T   M   Q   Y   E   L   K

181  GTG AAT GGA GAG CCA GTG GTC CTT CAC CTG GAA AAA AAT AAA GGA
61   V   N   G   E   P   V   V   L   H   L   E   K   N   K   G

226  CTT TTT TCA AAA GAT TAC AGT GAG ACT CAT TAT TCC CCT GAT GGC
76   L   F   S   K   D   Y   S   E   T   H   Y   S   P   D   G

271  AGA AAA ATT ACA ACA AAC CCT CCG GTT GAG GAT CAC TGC TAT TAT
91   R   K   I   T   T   Q   P   P   V   E   D   H   C   Y   Y
                   Zymogen propeptide domain
316  CAT GGA CGC ATC CAG AAT GAT GCT GAC TCA ACT GCA AGC ATC AGT
106  H   G   R   I   Q   N   D   A   D   S   T   A   S   I   S

361  GCA TGC AAT GGT TTG AAA GGA CAT TTC AAG CTT CAA GGG GAG ATG
121  A   C   N   G   L   K   G   H   F   K   L   Q   G   E   M

406  TAC CTT ATT GAA CCC TTG AAG CTT TCC GAC AGT GAA GCC CAT GCA
136  Y   L   I   E   P   L   K   L   S   D   S   E   A   H   A

451  GTC TTC AAA TGT GAA CAT ATA GAA AAG GAG GAT GAG GCC CCC AAA
151  V   F   K   C   E   H   I   E   K   E   D   E   A   P   K

496  ATG TGT GGG GTA ACC CAG ACT AAT TGG GAA TCA TAT GAG CCC ATC
166  M   C   G   V   T   Q   T   N   W   E   S   Y   E   P   I
Switch structure
541  AAA AAG GCC TCT CAG TTA GTT GTT ACT ACT TAT CAA AGA TAC GTT
181  K   K   A   S   Q   L   V   V   T   T   Y   Q   R   Y   V

586  GAG CTT GTC GTA GTT GCG GAC CAT AGA ATG GTC ACG AAA TAC AAT
196  E   L   V   V   V   A   D   H   R   M   V   T   K   Y   N

631  GGC AAT TTA ATT ATA ATA AGA ACA TGG GTA TAT GAA ATT TTC AAC
211  G   N   L   I   I   I   R   T   W   V   Y   E   I   F   N

676  ACT ATA AAT GAG ATT TAC CAA CGT ATG AAT ATT CAT GTA GCA CTG
226  T   I   N   E   I   Y   Q   R   M   N   I   H   V   A   L
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721  GTT GGC CTA GAA ATT TGG TCC AAC GGA GAT AAG ATT ATA GTG CAG
241  V   G   L   E   I   W   S   N   G   D   K   I   I   V   Q
                   Metalloprotease domain
766  ACA GCA GCG GCT GAT ACT TTG GAC TTA TTT GGA AAC TGG AGA GAG
256  T   A   A   A   V   T   L   D   L   F   G   N   W   R   E

811  ATA GAT TTG CTG AAG CGC AAA AGT CAT GAT AAT GCT CAG TTA CTC
271  I   D   L   L   K   R   K   S   H   D   N   A   Q   L   L

856  ACG CCC ATT GAC TTT GAT GGA CCA ACT ATA GGA TTG GCT TAT GTG
286  T   P   T   D   F   D   G   P   T   I   G   L   A   Y   V

901  GGC ACC ATG TGC GAC CCA AAG CGT TCT ACA GGA GTT GTC CAG GAT
301  G   T   M   C   D   P   K   R   S   T   G   V   V   Q   D

946  CAT AGC CCA ATA AAT CTT TTG GTT GCA GTT ACA ATG GCC CAT GAG
316  H   S   P   I   N   L   L   V   A   V   T   M   A   H   E

991  ATA GGT CAT AAT CTG GGC ATG CAT CAT GAC ACA AGT TAC TGT TCT
331  I   G   H   N   L   G   M   H   H   D   T   S   Y   C   S
Zinc binding and catalytic motif
1036 TGC GGT GGT ACT GCA TGC ATT ATG TCT CCC GTG ATA AGC CAT GAA

346  C   G   G   T   A   C   I   M   S   P   V   I   S   H   E
                   Met-turn
1081 CCT TCC AAA TTT TTC AGC TAT TGT AGT TAT ATC CAA TGT TGG GAC
361  P   S   K   F   F   S   Y   C   S   Y   I   Q   C   W   D

1126 TAT ATT AAG AAT CGG AAC CCA CAA TGC ATT CTC AAT AAA CCC TTG
376  Y   I   K   N   R   N   P   Q   C   I   L   N   K   P   L

1171 AGA ACA GAT ACT GTT TCA ACT CCA GTT TCT GGA AAT GAA CTT TTG
391  R   T   D   T   V   S   T   P   V   S   G   N   E   L   L
           spacer region
1216 GAG GCG GGA GAA GAA TGT GAC TGT GAC TCT CCT GGA AAT CCG TGC
406  E   A   G   E   E   C   D   C   D   S   P   G   N   P   C
                 Disintegrin domain
1261 TGC GAT GCT GCA ACC TGT AAA CTG AGA CCA GGG GCA CAG TGT GCA
421  C   D   A   A   T   C   K   L   R   P   G   A   Q   C   A

1306 GAA GGA CTG TGT TGT GAG CAG TGC AGA TTT ATG AAA GAA GGA ACA
436  E   G   L   C   C   E   Q   C   R   F   M   K   E   G   T
1351 GTA TGC CGG ATA GCA AGG GGT GAT GAC ATG GAT GAT TAC TGC AAT
451  V   C   R   I   A   R   G   D   D   M   D   D   Y   C   N

1396 GGC ATA TCT GCT GGC TGT CCC AGA AAT CCC TTC CAT GCC TGA
466  G   I   S   A   G   C   P   R   N   P   F   H   A   *
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Fig.1 cDNA and deduced amino acid sequence of pro-Ussurin that is the precursor of Metalloproteinases/disintegrin from Gloydius ussuriensis venom

From the initial coden, the putative signal peptide is indicated by a waved underline, the zymogen propeptide is indicated by a single underline, the metalloproteinase domain is indicated by a dash line, the space region is indicated by a dotted line, the disintegrin domain is indicated by a thick underline, the conserved switch structure, zinc binding and catalytic motif, met-turn and RGD motif are indicated by a gray color

Figure 2 shows the predicted amino acid sequence of Ussurin was analyzed for similarities to other SVMPS sequence in GenBank. It was 76% homologous to bothrostatin precursor of Bothrops jararaca, 76% homologous to metalloprotease of Gloydius halys, 75% homologous to hemorrhagic metalloproteinase HT-E of crotalus atrox, 72% homologous to aculysin-2 of Deinagkistrodon acutus.

3 Discussion

SVMPS have presented strong influence on hemorrhage, platelet aggregation and extracellular matrices (ECMS). According the molecular mass, the SVMPS can be divided into P-Ⅰ, P-Ⅱ, P-Ⅲ and P-Ⅳ. The marked structure to differ from one another is the following domain on the carboxyl side of the metalloproteinase domain. Basing on the cDNA size and deduced molecular mass, Ussurin contains both a proteinase domain and disintegrin-like domain, which belongs to P-Ⅱ class.

Mechanisms whether P-Ⅱ precursor generate a P-Ⅰ or P-Ⅱ proteinase after post-translation processing are still not clear. However, reports show that the disintegrin domain releases from precursor autoproteolyticall *in vivo* and suggest that P-Ⅰ class to be the product of processed P-Ⅱ precursor. 5′cDNA sequence for the Ussurin and other SVMPs is highly conserved and code for 18 amino acids domain of signal peptide. It is hypothesized that processing occurs between Gly^{18} and Ser^{19} to release the signal sequence of 18-residue stretch of amino acids.

The zymogen domain presented high conservative, the homologous of this domain is as high as 96% when blasting with GenBank. The identical structure means the single function in the P-Ⅱ precursor. The cysteine residue ahead starting amino acid sequence of matalloproteinase combines with amino acids before and behind cysteine and conserves amino acid (PKMCGVT) motif. It is hypothesized that the region may be function as cysteine switch. SVMPS belongs to reprolysin subfamily of the metzincin superfamily. All of them have a long conservation HEXXHXXGXXH of zinc binding region and comprise the catalytic center. Another significantly homologous sequence is CIMXP in matalloproteinase, named met-turn, and might be a switch structure in activation procession of metalloproteinase by comparing with matrix metalloproteinase and is presumed to involved in zinc binding activity. Disintegrins contain a highly conserved RGD sequence and function an antogonist of RGD-dependant integrins adhesion.

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u:   1 MIQVLLVTICLAVFPYQGSSIILESGNVNDYEVVYPRKVCALPKEAVQPKYEDTMQYELK 60
B:   1  MIEVLLVTICLAAFPYQGSSIILESGNVNDYEVIYPRKVTALPKGAVQPKYEDAMQYELK 60
G:   1  MIQVLLVTICLAAFPYQGSSMILESGNVNDYEVVYPQKVPALPKGAVPQKYEDAMQYEFK 60
D:   1  MIQVLLVTICLAAFPYQGSSIILESGKVNDYEVVYPQRLAPLPEGAVQQKYEDTMQYEFK 60
A:   1  MIQVLLVTLCLAAFPYQGSSIILESGNVNDYEVLYPQKVTALPKGAVQPKYEDTMQYEFK 60

u:  61  VNGEPVVLHLEKNKGLFSKDYSETHYSPDGRKITTQPPVEDHCYYHGRIQNDADSTASIS 120
B:  61  VNGEPVVLHLEKNKGLFSKDYSETHYSPDGRKITTNPPVEDHCYYHGRIENDADSTASIS 120
G:  61  VNGEPVVLHLEKNKGLFSKDYSETHYSPDGRKITTNPPVEDHCYYHGHIQNDADSTASIS 120
D:  61  VNGEPVVLHLEKNKGLFSKDYSEIHYSPDGRRITTHPLVEDHCYYRGHIRNDADSTASIS 120
A:  61  VNGEPVVLHLEKNKGLFSKDYSETHYSSDGRKITTNPPVEDHCYYHGRIQNDADSTASIS 120

u: 121 ACNGLKGHFKLQGEMYLIEPLKLSDSEAHAVFKCEHIEKEDEAPKMCGVTQTNWESYEPI 180
B: 121 ACNGLKGHFKLQGETYLIEPLKLSDSEAHAVFKFENVEKEDEAPKMCGVTQ-NWESYEPI 179
G: 121 ACNGLKGHFKHQGEMYLIEPLKLSDSEAHAVYKYENVEKEDEAPKMCGVTQTNWKSDEPI 180
D: 121 ACHGLKGHFKLRGETYLIEPMKISNSEAHAVYKYENVEKEDEAHKMCGVTQ-NWESYEPI 179
A: 121 ACNGLKGHFKLQGETYLIEPLKLSDSEAHAVYKYENVEKEDEAPKMCGVTQTNWESDEPI 180

u: 181 KKASQLVVT-----TYQRYVELVVVADHRMTAKANGNLIIINTRVYEIFNTINEIYQRMNIHVA 239
B: 180 KKASQSNLT----PEHQRYIELFLVVDHGMFMKYNGNSDKIRRRIHQMVNIMKEAYRYLYIDIA 239
G: 181 -KASQLVVTAEQQRFPQRYIELVVVADHGMFTKYDSNLDTITTWVHELVNNINEFYRSLNVRVS 244
D: 180 KKASQLIVST----EQQRYMEIVIVVDHSMVKKYNGDSDKIKAWVYEMINTITESYRYLYIDII 239
A: 181 KKASQLNLTPEQQRFPQRYIELVVVADHRMFTKYNGNLNTIRIWVHELVNTMNVFYRPLNIRVS 245

u: 240 LVGLEIWSNGDKIIVQSSADVTLDLFGTWGEIDLLKRKSHDNAQLLTPTDFDGPTIGLAY 299
B: 240 LTGVEIWSNKDMINVQPAAPQTLDSFGEWRKTDLLNRKSHDNAQLLTSTDFKDQTIGLAY 299
G: 245 LTELEIWSNQDLINVQSAAADTLEAFGDWRETDLLNRISHDNAQLLTTIDLDGNTIGLAH 304
D: 240 LSGLEIWSEKDLINVETSAENTLKSFGEWRAKDLIHRISHDNAQLLTATDLDGPTIGLAY 299
A: 246 LTDLEVWSDQDLINVQPAAADTLEAFGDWRETVLLNRISHDNAQLLTAIELDGETIGLAN 305

u: 300 VGTMCDPKRSTGVVQDHSPINLLVAVTMAHEIGHNLGMHHDTSYCSCGGTACIMSPVISH 359
B: 300 WGSMCDPKRSTAVIEDHSETDLLVAVTMAHELGHNLGIRHDTGSCSCGGYSCIMAPVISH 359
G: 305 VGTMCDPKYSVGIVQDHSAINLLVAVTMAHELGHNLGMDHDGNQCHCGANSSVMGDVLRV 363
D: 300 VASMCDPKRSVGIVQDHSSVNRLVAITLAHEMAHNLGVRHDEGSCSCGSGYCIMSPVINP 359
A: 306 RGTMCDPKLSTGIVQDHSAINLWVAVTMAHEMGHNLGISHDGNQCHCDANSCIMSEELRE 365

u: 360 EPSKKNSYCSYIQCWDYINYRNPQCILNKPLRTDTVSTPVSGNELLEAGEECDCDSPGNP 419
B: 360 DIAKYFSDCSYIQCWDFIMKDNPQCILNKQLRTDTVSTPVSGKN-FGAGEECDCGTPGNP 418
G: 364 GVSYEFSDCNENEYQTYVTDHNPQCILNEPLRTDTVSTPVSGNELLEAGVECDCGAPANP 423
D: 360 DAMKYFSDCSYIQCWDYIMKENPPCILNKPLRTDTVSTPVSGNELLEAGKDYDRDSSANP 419
A: 366 QLSFEFSDCSQNQYQTYLTDHNPQCMLNEPLRTDIVSTPVSGNELLETGEESDFDAPANP 425

u: 420 CCDAATCKLRPGAQCAEGLCCEQCRFMKEGTVCRIARGDDMDDYCNGISAGCPRNPFHA 478
B: 419 CCDAVTCKLRPGAQCAEGLCCDQCRFMKEGTVCRRARGDDMDDYCNGISAGCPRNPFHA 477
G: 425 CCDAATCKLRPGAQCAEGLCCDQCRFMKEGTICRMARGDDMDDYCNGISAGCPRNPFHA 483
D: 420 CCDAATCKLNQGAQCTAGPCCDQCRFKEEGTICRRARGDDLDDYCNGISADCPRNPYHA 479
A: 426 CCDAATCKLTTGSQCADGLCCDQCKFMKEGTVCRRARGDDLDDYCNGISAGCPRNPFHA 485
U= Ussurin

B = (Bothrops jararaca ) Identities = 375/479 (78%)

G = (Gloydius halys) Identities = 385/505 (76%),

D = (Deinagkistrodon acutus) Identities = 347/480 (72%)

A = (Agkistrodon contortrix  contortrix)  Identities = 363/483 (75%),
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Fig.2 Comparison of the amino acid sequence deduced from pro-Ussurin cDNA with genes for metalloproteinase of other species
The conserved cysteines are in gray color

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