行政院國家科學委員會專題研究計畫　成果報告

心肌衰竭病程中細胞骨骼蛋白質結構變化之研究

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執行期間：九十一年八月一日至九十二年七月三十一日

計畫主持人：許榮彬

共同主持人：朱樹勳 蔡有光

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執行單位：國立台灣大學醫學院附設醫院外科

中　華　民　國　九十二　年　十　月　三十一　日

一、中英文摘要：

（一）中文摘要。（五百字以內）

心臟衰竭的原因，或是因為心臟本身功能不足，或是由於其他身體組織需求過高。前者之主因有冠狀動脈疾病、心臟瓣膜疾病、高血壓，後者則主要肇因於不明原因的心肌病變。心臟衰竭病人的治療費用，佔整個醫療資源的比例，有逐漸增加的趨勢。不當的治療，勢將導致死亡率與致病率的上升，形成社會沉重的負擔。

然而心臟衰竭的基本生理學，至今仍不甚瞭解。經由分生實驗顯示，似乎有各種機轉參與其中。近來更有實驗顯示，心肌內細胞骨骼之變化，可能與心臟衰竭病程之演變有關。以往，確有許多實驗研究細胞之骨骼蛋白修飾基之變化，並試圖了解這些修飾基與骨骼蛋白系統功能的關聯。然而，這些實驗使用的材料範圍甚廣，包括許多不同的細胞與組織，至於心肌內細胞骨骼蛋白修飾基的研究，目前仍未有任何有系統的探討。本計劃運用動物實驗之模式，以創新之蛋白質分析技術來探究心臟衰竭的病程當中、心肌細胞內細胞骨骼蛋白結構之變化。我們在二維電泳的實驗中發現，某些心肌蛋白質的表現會增加，而某些會受到抑制。而受到抑制的蛋白質中，有兩個是蛋白酶的抑制蛋白，似乎意味著蛋白酶的活性增加與心肌衰竭的發生有關。而這些初步的成果也證明我們的實驗系統已完備，可以用來研究細胞骨骼蛋白的結構性變化。

關鍵詞：細胞骨骼, 心肌衰竭, 蛋白質修飾基, 液相層析–串聯式質譜儀術

（二）英文摘要。（五百字以內）

Heart failure is defined as the pathologic state of impaired cardiac function rendering the heart unable to maintain an output sufficient for the metabolic requirements of the body's tissues and organs. Heart failure refractory to medical treatment consumes an ever increasing amount of health care resources. Heart failure is a major public health issue. It is a major source of morbidity and mortality, is expensive to treat and is frequently treated inappropriately.

Although progress has been made in our understanding of the basic pathophysiology of heart failure, many mechanisms involved remain unclear. Applications of the techniques of molecular and cell biology to the study of heart failure are providing new insights into the mechanisms responsible for this important clinical problems. The basic mechanisms involved include beta-adrenergic recepters, cytokines, nitric oxide, and apotosis. There is some evidence indicating that cytoskeletal proteins like tubulin and desmin are also crucial for development of pathologic processed leading to heart failure. Here, using an animal model of LV pressure overload, we would like to investigate the in vivo structural changes of cytoskeletal proteins during the evolution to heart failure. The two-dimensional gel electrophoresis revealed that a small group of proteins are differentially expressed between normal rats and those with one-month cardiac pressure overload. Thus far, we found that the expression of two proteinase inhibitors was severely reduced. It should be intriguing to explore whether enhanced proteinase activity might be involved in functional changes related to heart failure. Nevertheless, these preliminary data showed that this model system should be of great use in the studies about the cytoskeletal changes during the disease process.

Keywords：cytoskeleton, heart failure, post-translational modification, LC/MS/MS

二、報告內容：

前言

Heart failure refractory to medical treatment consumes an ever increasing amount of health care resources (O'Connell *et al*, 1994). Heart failure is a major public health issue. It is a major source of morbidity and mortality, is expensive to treat and is frequently treated inappropriately. Heart failure affects approximately 2.5% of the population over 45 years (Ho *et al*, 1993), with 5-year survival rates of 25% in men and 38% in women (Ho *et al*, 1993a; Ho *et al*, 1993b). Heart failure is associated with a shorter life expectancy than that of many common malignancies (Ho *et al*, 1993b). Several pharmacologic and surgical approaches to the treatment of refractory failure are already being clinically applied, evaluated in clinical trials, or are at various stages of development. Currently available approaches for patients with refractory heart failure include intravenous inotropic therapy, partial left ventriculectomy, dynamic cardiomyoplasty, mechanical circulatory support and heart transplantation. However, heart transplantation still remains a conventional treatment for end-stage heart failure. In the 21st century, the population of patients who progress to the end-stage heart failure will continue to grow. The prevalence of heart failure in Taiwan is also rising due to the increase of the overall age of the population, the success of medical therapy for previously acutely devastating illnesses such as acute myocardial infarction, and the application of therapies known to prolong the life of patients with heart failure. The cost of management of heart failure is difficult to assess. The economic burden of cardiovascular disease, which was the fourth leading cause of mortality in Taiwan in 1998, will continue to increase in the future.

Although progress has been made in our understanding of the basic pathophysiology of heart failure, many mechanisms involved remain unclear. Applications of the techniques of molecular and cell biology to the study of heart failure are providing new insights into the mechanisms responsible for this important clinical problems. The basic mechanisms involved include beta-adrenergic recepters, cytokines, nitric oxide, and apotosis. Myocardial beta-adrenoceptors are down-regulated in severe heart failure and beta-blockers therapy for dilated or ischemic cardiomyopathy is now an accepted and effective treatment. Recent evidences suggest that an inflammatory progress may be involved in the development of heart failure. Cytokines, including tumor necrosis factor-alpha and interleukin-6, and nitric oxide play a major role in this inflammatory process. Previous studies (Birks *et al.*, 1997) have shown that tumor necrosis factor-alpha, interleukin-6, and inducible nitric oxide synthetases are increased in patients of heart failure. Cytokines may be produced in heart failure, and induce inducible nitric oxide synthetases, thus resulting in the production of large amount of nitric oxide, which may act as a negative inotropy. Apoptosis, or programmed cell death, may also play some role in the process of cardiomyocyte degeneration in patients with end-stage heart failure.

研究目的

Because of increasing number of heart failure patients and limited role of medical and surgical therapies, it is imperative to understand the pathogenesis of heart failure and prevent its development. Here, using an animal model of LV pressure overload, we have investigated the *in vivo* structural changes of cytoskeletal proteins during the evolution to heart failure.

研究方法

Animals— Male Sprague-Dawley rats weighing 275-325 g (7-9 wk of age; Harlan Sprague Dawley, Indianapolis, IN) have been used in all experiments. Rats were housed two per cage with free access to food and water.

Surgical Induction of Pressure Overload— Rats were randomly assigned to groups [aortic constricted (AC) and sham operated (SO)] and time points (3, 10, 30, or 60 days; n = 8 rats/group at each time point). Rats were anesthetized by ketamine hydrochloride plus acepromazine maleate (90 and 0.02 mg/kg ip, respectively; Fort Dodge Laboratories, Fort Dodge, IA) and prepared for surgery under aseptic conditions. After a midline abdominal laparotomy, pressure overload was induced by suprarenal abdominal aortic constriction using a tantalum Weck hemoclip (Pilling Weck, Research Triangle Park, NC) tightened to the diameter of a 22-gauge needle. Control rats underwent sham surgery consisting of midline laparotomy and isolation of the suprarenal abdominal aorta without constriction. The muscle was sutured, and the skin was closed using surgical wound clips. On the final day of the study, each rat was examined to verify the location of the hemoclip, and both kidneys were weighed to identify the presence of renal atrophy.

Extraction of myocardial proteins. Equal volume of 2% SDS solution containing 1 mM Tris and 10 mM dithiothreitol was added to the rat heart specimen. The mixture was homogenized in a min-grinder and then incubated at 100° for 10 min. The solution was sonicated 10 × 3 sec and pelleted by high speed centrifugation for 10 min. The supernatant was collected and stored at -20°.

Two-dimensional gel electrophoresis. Two-dimensional electrophoresis was performed using a nonlinear immobilized pH gradient (range 4-7) in the first dimension with Immobiline strips (Pharmacia, Uppsala, Sweden). Tissue lysates of myocardium were pelleted by low speed centrifugation and resuspended in 10X volume of 8 M urea, 2% (w/v) TritonX-100, 40 mM Tris base, 10 mM DTT. A SDS-PAGE 4-15% (w/v) acrylamide gradient was used in second dimension. For Western blot analysis, the gel was subjected to protein transfer followed by immnuolabeling. The apparent pI and MW were then estimated. For LC/MS/MS analysis, the gel was developed by silver staining and desired

proteins were located.

In-gel digestion— The proteins on the 2D gel were excised and prepared for LC/MS/MS analysis. The gel piece containing polypeptides was soaked in 25 mM NH_4HCO_3 for 10 min and then in 25 mM NH4HCO3/50% acetonitrile for 10 min. After drying in a Speed-Vac (Savant), the gel was incubated in 100 ml of 2% β-mercaptoethanol/25 mM NH_4HCO_3 for 20 min at room temperature and at dark. The same volume of 10% 4-vinylpyridine in NH_4HCO_3/50% acetonitrile was added for cysteine alkylation. After a 20-min incubation, the gel was soaked in 1 ml of 25 mM NH4HCO3 for 10 min. After being washed in 25 mM NH_4HCO_3/50% acetonitrile for 10 min, the gel was dried and then incubated with 25 mM NH_4HCO_3 containing 50 ng of modified trypsin (Promega) or other appropriate endoproteinases overnight (~18 h). The tryptic digest was removed from the gel, which was extracted with 200 μl of 0.1% formic acid. These two fractions were combined together and then were dried in a Speed-Vac and was then kept at -20° for storage. It was resuspended in 0.1% formic acid immediately before LC/MS/MS analysis.

LC/MS/MS analysis— The protein were identified using the method described in Yang al 2002. Electrospray mass spectrometry is performed using a Finnigan Mat LCQ ion trap mass spectrometer interfaced with an ABI 140D HPLC (Perkin-Elmer). A 150 3 0.5 mm PE Brownlee C18 column (Perkin-Elmer) (5 mm particle diameter, 300 Å pore size) with mobile phases of A (0.1% formic acid in water) and B (0.085% formic acid in acetonitrile) are used. The peptides are eluted at a flow rate of 5 μl/min with an acetonitrile gradient, which increases B from 5% to 65% in 45 min. The spectra for the eluate are acquired as successive sets of three scan modes. The MS scan determines the intensity of the ions in the m/z range of 395 to 1605, and a specific ion was selected for zoom scan and MS/MS scan. The former examines the charge number of the selected ion and the latter acquires the spectrum (CID spectrum or MS/MS spectrum) for the fragment ions derived by collision-induced dissociation. The MS/MS spectra were matched with Sequest (ThermoFinnigan) against the non-redundant sequence databases containing proteins from all species.

結果與討論

In order to study the roles of cytoskeleton in pathogenesis of heart failure, we set up a rat model to carry out a controlled experiment. The rats were subjected to constriction procedure on the abdominal aorta. These rats have been kept for different times and then subjected to hemodynamic studies and followed by harvesting of the myocardium. Our preliminary data showed that most of these animals may survive the operation. While the hemodynamic data are yet to be analyzed, we first examine the proteomic changes in myocardium as pressure overload is applied.

The proteins in collected myocardium were extracted and then subjected to two-dimensional gel electrophoresis. The gels were developed by silver staining method. For example, there are more than a thousand proteins from normal myocardium resolved on a regular 2-D gel (Fig. 1). Likewise, we also analyzed the myocardial proteins from rat under pressure overload stress for a month (Fig. 2). The gel patterns of these two samples were compared mostly with visual inspection. There were some protein spots differently expressed in terms of their staining intensities. These proteins have been subjected to protein identification analysis to reveal what proteins are differentially expressed upon cardiac pressure overload.

For example, we found that the expression of a1 proteinase inhibitor III and contrapsin-like protease inhibitor 1 are suppressed in rat with the pressure overload. Both proteins have functions related to protein degradation, namely inhibition of protein degradation. It is supposed that lowered activities of these proteins might result in proteinase hyperactivity. It is very intriguing to examine whether the expected proteinase hyperactivity is related to the progressive change of myocardium.

Nevertheless, we demonstrate here that we have successfully established the rat model for studying cardiac pressure overload and the secondary hear failure. As we acquire the information through hemodynamic analysis, this enables us to identify the differentially expressed proteins during this process. The cytoskeletal proteins are also supposed to be present in the 2-D gel. These proteins will be our major experimental targets in the near future.

圖表

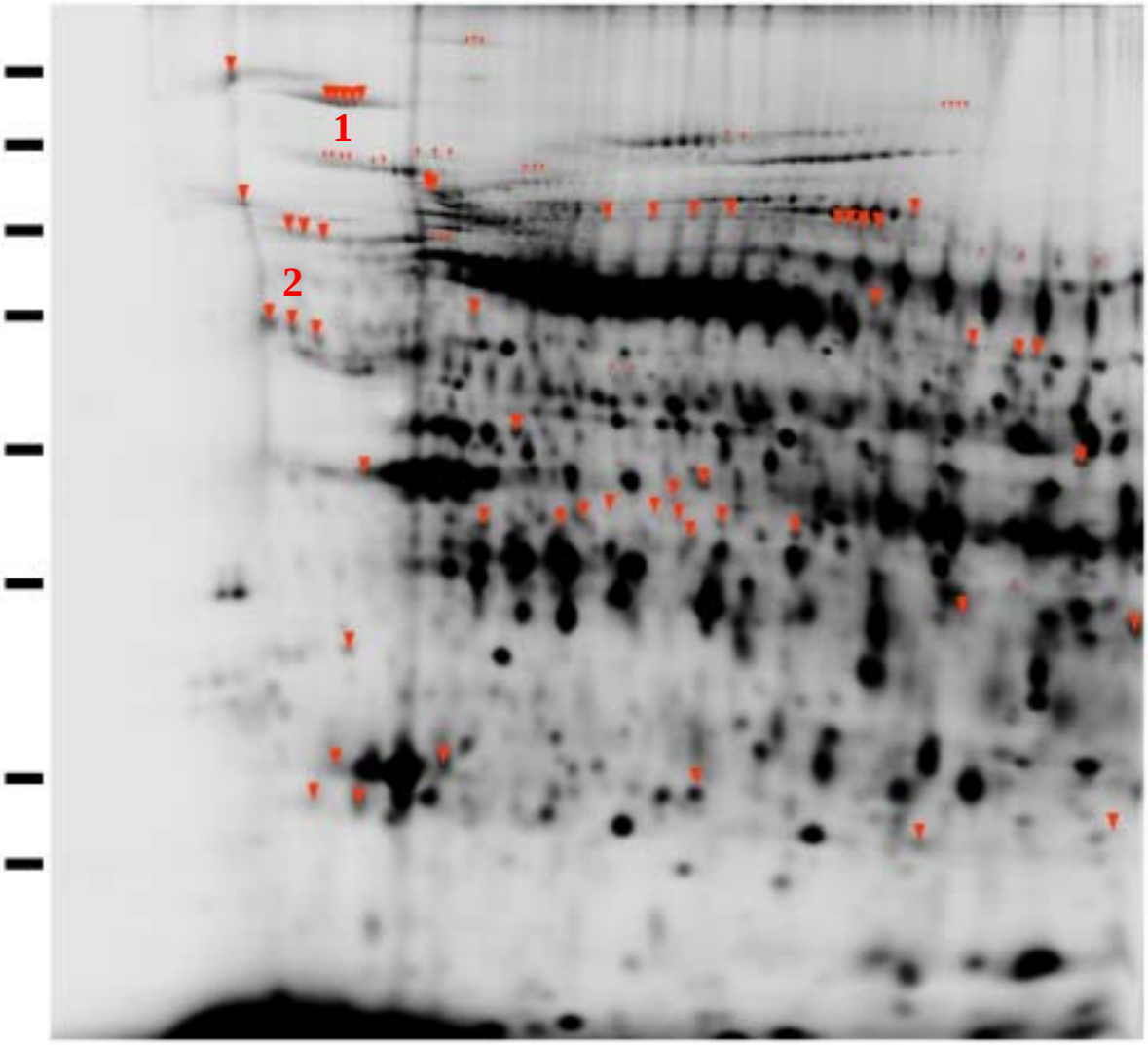

Fig. 1. Two-dimensional gel electrophoretic analysis of myocardial protein from normal rat. The bars at left indicate the position of molecular weight markers. Those proteins whose expression becomes depressed upon cardiac pressure overload are indicated by red arrow heads. The numbers in the gels denote the positions of proteins spots that have been analyzed by LC-MS/MS analysis.

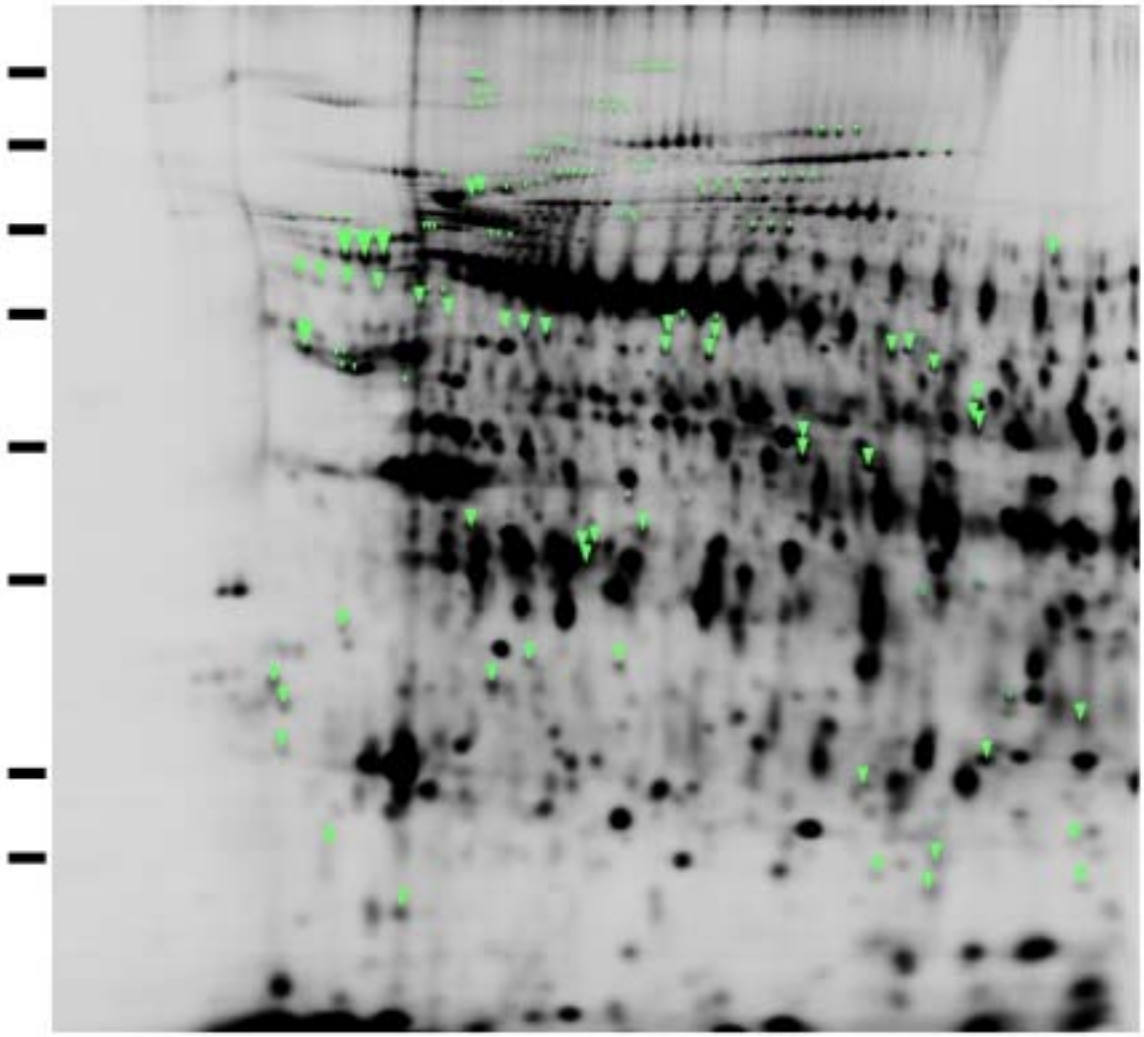

Fig. 2. Two-dimensional gel electrophoretic analysis of myocardial protein from rat with pressure overload for a month. The bars at left indicate the position of molecular weight markers. Those proteins whose expression becomes enhanced upon cardiac pressure overload are indicated by green arrow heads.

MKKDREAQLC LFSALLAFLP FASLLNGNSK YMVLVPSQLY TETPEKICLH LYHLNETVTV TASLISQRGT RKLFDELVVD

KDLFHCVSFT IPRLPSSEEE ESLDINIEGA KHKFSERRVV LVKNKESVVF VQTDKPMYKP GQSVKFRVVS MDKNLHPLNE

LFPLAYIEDP KMNRIMQWQD VKTENGLKQL SFSLSAEPIQ GPYKIVILKQ SGVKEEHSFT VMEFVLPRFG VDVKVPNAIS

VYDEIINVTA CATYTYGKPV PGHVKISLCH GNPTFSSETK SGCKEEDSRL DNNGCSTQEV NITEFQLKEN YLKMHQAFHV

NATVTEEGTG SEFSGSGRIE VERTRNKFLF LKADSHFRHG IPFFVKVRLV DIKGDPIPNE QVLIKARDAG YTNATTTDQH

GLAKFSIDTN GISDYSLNIK VYHKEESSCI HSSCTAERHA EAHHTAYAVY SLSKSYIYLD TEAGVLPCNQ IHTVQAHFIL

KGQVLGVLQQ IVFHYLVMAQ GSILQTGNHT HQVEPGESQV QGNFALEIPV EFSMVPVAKM LIYTILPDGE VIADSVKFQV

EKCLRNKVHL SFSPSQSLPA SQTHMRVTAS PQSLCGLRAV DQSVLLQKPE AELSPSLIYD LPGMQDSNFI ASSNDPFEDE

DYCLMYQPIA REKDVYRYVR ETGLMAFTNL KIKLPTYCNT DYDMVPLAVP AVALDSSTDR GMYESLPVVA VKSPLPQEPP

RKDPPPKDPV IETIRNYFPE TWIWDLVTVN SSGVTELEMT VPDTITEWKA GALCLSNDTG LGLSSVASFQ AFQPFFVELT

MPYSVIRGEA FTLKATVLNY LPTSLPMAVL LEASPDFTAV PVENNQDSYC LGANGRHTSS WLVTPKSLGN VNFSVSAEAR

QSPGPCGSEV ATVPETGRKD TVVKVLIVEP EGIKKEHTFS SLLCASDAEL SETLSLLLPP TVVKDSARAH FSVMGDILSS

AIKNTQNLIQ MPYGCGEQNM VLFAPNIYVL KYLNETQQLT EKIKSKALGY LRAGYQRELN YKHKDGSYSA FGDHNGQGQG

NTWLTAFVLK SFAQARAFIF IDESHITDAF TWLSKQQKDS GCFRSSGSLL NNAMKGGVDD EITLSAYITM ALLESSLPDT

DPVVSKALSC LESSWENIEQ GGNGSFVYTK ALMAYAFALA GNQEKRNEIL KSLDKEAIKE DNSIHWERPQ KPTKSEGYLY

TPQASSAEVE MSAYVVLARL TAQPAPSPED LALSMGTIKW LTKQQNSYGG FSSTQDTVVA LDALSKYGAA TFSKSQKTPS

VTVQSSGSFS QKFQVDKSNR LLLQQVSLPY IPGNYTVSVS GEGCVYAQTT LRYNVPLEKQ QPAFALKVQT VPLTCNNPKG

QNSFQISLEI SYMGSRPASN MVIADVKMLS GFIPLKPTVK KLERLGHVSR TEVTTNNVLL YLDQVTNQTL SFSFIIQQDI

PVKNLQPAIV KVYDYYETDE VAFAEYSSPC SSDDQNV

Fig. 3. The amino acid sequence of the spot 1 protein, α1 proteinase inhibitor III. The sequences of the peptide identified by LC/MS/MS method are underlined and marker by red letter.

MAFIAALGLL MAGICPAVLC DGILGR**DTLP HEDQGK**GRQL HSLTLASINT DFTLSLYKKL ALRNPDKNVV FSPLSISAAL

AILSLGAKDS TMEEILEVLK FNLTEITEEE IHQGFGHLLQ RLSQPEDQAE INTGSALFID K**EQPILSEFQ EK**TR**ALYQAE**

AFVADFKQCN EAKKFINDYV SNQTQGK**IAE LFSELDER**TS MVLVNYLLFK GKWKVPFNPN DTFESEFYLD EKRSVKVPMM

KIKDLTTPYI RDEELSCSVL ELKYTGNASA LFILPDQGK**M QQVESSLQPE TLKK**WKDSLR PRIISELRMP KFSISTDYNL

EEVLPELGIR **KIFSQQADLS R**ITGTK**NLHV SQVVHK**AVLD VDETGTEGAA ATAVTAALKS LPQTIPLLNF NRPFMLVITD

NNGQSVFFMG KVTNPM

Fig. 4. The amino acid sequence of the spot 2 protein, contrapsin-like protease inhibitor 1. The sequences of the peptide identified by LC/MS/MS method are underlined and marker by red letter.