BIOLOGIJA. 2006. Nr. 3. P. 53–58

Investigation of human heart tissue extracts by spectroscopic methods

J. Venius[1],

D. Labeikytė[2],

E. Žurauskas[3],

V. Strazdaitė[1],

S. Bagdonas[1],

R. Rotomskis[1]

[1] Laser Research Center, Vilnius University, Vilnius, Lithuania

[2] Department of Biochemistry and Biophysics, Vilnius University, Lithuania

[3] Department of Medicine, Vilnius University, Vilnius, Lithuania

The conduction system of the heart (HCS) is a type of muscular tissue which generates and transmits bioelectrical impulses. During surgical operations it is possible to harm HCS, since the common origin makes it hardly discernible for an unaided eye in the surroundings of ordinary myocardium (MC) or endocardium. The aim of this study was to reveal the protein composition differences between His bundle (HB) and MC tissues and to determine the distribution of fluorophores in these tissues. This in turn would help to visualize HCS by means of optical-fluorescence biopsy. It has been shown that fluorescence of the soluble fractions of heart tissues is mainly determined by tryptophan (W) and tyrosine (Y) residue emission, while fluorophores, responsible for the fluorescence in the visible region, were found to be hardly extractable from tissues and precipitated out as the insoluble fraction. According to SDS-PAGE, some protein groups specific to MC and HB were revealed. Some SDS-PAGE gel sections containing certain proteins of heart tissue fractions were investigated by spectroscopic methods. The results indicated that proteins of the same weight extracted from different heart tissues exhibit different fluorescence spectra.

Key words: heart conduction system, His bundle, fluorescence spectroscopy, electrophoresis, protein composition

Abbreviations: heart conduction system, HCS; sinoatrial node, SA; atrioventricular bundle of His, HB; myocardium, MC; connective tissue, CA; denaturing polyacrilamide gelelectrophoresis, SDS-PAGE; tyrosine, Y; tryptophan, W.

INTRODUCTION

The heart beats approximately three billion times during the lifespan of an average person. Each of these cycles of contraction depends on specialized cardiac tissues involved in the rhythmic generation and the coordinated spread of electrical excitation. Collectively, these tissues are known as the pacemaking and conduction system. Since the conduction system is vital for generating and synchronizing the heartbeat, its dysfunction caused by various diseases or postoperational complications can be a direct cause of cardiac conduction disturbance, arrhythmias and sudden cardiac death [1]. The conduction system comprises two main parts: the sinoatrial (SA) node and the atrioventricular system. The latter begins from the atrioventricular node, then extends as the bundle of His (HB) that ramifies to the left and right branches and ends in the subendocardium of the corresponding ventricles. The heart beat signal originates in the SA node and then through the atrioventricular branches spreads to the different parts of the heart, initiating the contraction of the ventricles. The conduction system of the heart (HCS) plays a totally different role from the myocardium (MC) and has different electric characteristics – an impulse through HB is propagated about ten times faster than in the myocardium [2]. HB and its branches resemble ordinary MC and visually are hardly distinguishable, therefore there is a possibility to harm HCS during surgical intervention, what may result in a dangerous obstruction of heart functionality. To avoid such kind of disturbances, it is necessary to find a method for the visualization of HCS.

Various spectroscopical methods could be applied to study biological objects and the differentiation between them. Fluorescence reflects the intrinsic features of the tissue, therefore it should be useful for the differentiation between those tissues being otherwise visually indiscernible. There are only few reports concerning the studies performed on HCS by using the method of fluorescence spectroscopy. During their studies on human heart tissues, Perk et al. have shown that under excitation at 337 nm the fluorescence intensity from AV and

Corresponding author: Jonas Venius, Laser Research Center, Vilnius University, Saulėtekio 9, c. 3, LT-10222 Vilnius, Lithuania. E-mail: Jonas.Venius@ff.vu.lt

SA nodes in the blue region was higher than that from ventricular endocardium and approximately the same as from atrial endocardium [3]. Other researchers investigated myocardial tissue of sheep hearts and showed that atriums fluoresce more intensively than ventricles [4]. Collagen and elastin were found to be the main fluorophores characterising the fluorescence of the myocardial tissue [5].

The most significant difference in propagation velocities of a bioimpulse has been observed between HB and MC tissues, implying that these two muscular tissues should have the highest qualitative or quantitative composition differences or even different structure. The aim of this study was to investigate protein composition differences in the specimens of HB and MC and to determine the distribution of characteristic fluorophores in these tissues.

MATERIALS AND METHODS

Tissues and morphology

Samples of the heart tissues were randomly chosen from autopsy cases of different age without respect of pathology and prepared at the National Centre of Pathology. The samples of His bundle tissues were taken by micropreparation method [6]. Since HB is embedded in connective tissue (CT), both cardiac muscle and valve samples were taken as control tissues. All samples were stored at – 22 °C.

The morphology of the specialized conducting cells varies greatly in different components of the conduction system. Such variations are related to the degree to which these cells differ from ordinary working myocytes, particularly in their size and content of myofibrils and mitochondria (Fig. 1). Specific nodal cells in the proximal part of the atrioventricular node have a single nucleus and are connected by simple junctional complexes containing only a few gap junctions. These cells are abundant in glycogen and contain poorly organized contractile material that does not form myofibrillar insertion sites at the sarcolemma. In the central part of the atrioventricular node, cells have smaller diameters and are organized into fascicles separated by connective tissue. Junctional structures are found mostly at the ends of the cells. In the distal region atrioventricular nodal cells become wider, contain more clear spaces in their sarcoplasm, and are more closely associated with each other. Cells in a His bundle are arranged in parallel, are small and contain relatively few myofibrils. The cellular population in the bundle branches is inhomogeneous, and Purkinje fibers are found in close association with smaller cells, which variously resemble atrioventricular nodal cells, working myocytes, or transitional cells [7].

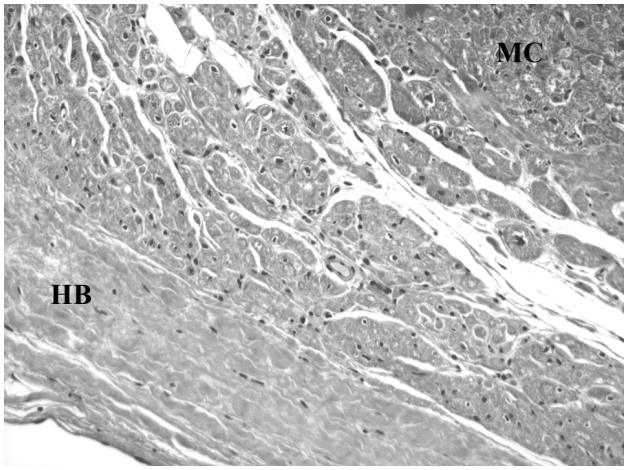

Fig. 1. Atrioventricular bundle of His is a compact cord. It resembles ordinary myocardium (stained with H&E, ×100).

Tissue disruption and preparation of extracts

Tissues (kept on ice) were weighed, chopped into small (1–5 mm) pieces with a scalpel, washed three times with ice-cold 0.9% KCl, and homogenized within tenfold volume excess of 5 mM Tris homogenization buffer containing 250 mM sucrose and 2 mM EGTA (pH 7.7 at 2°C), using a mini glass Potter homogenizer (0.1–2 ml) with 10–15 strokes. The homogenates were centrifuged for 5 min at 5000 *g*, the supernatant was collected (soluble fraction I) and the pellet was then homogenized within a tenfold volume excess of a buffer which consisted of 5 mM Tris, 180 mM KCl, 8 M urea, 2 mM EGTA (pH 7.7 at 2 °C) as before. The resulting supernatant was collected (soluble fraction II) and the pellet was homogenized within a fivefold volume excess of a buffer containing 50 mM Tris, pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 5% (w/v) 2-mercaptaethanol and centrifuged again. The resulting supernatant represented the soluble fraction III. The three different extracts from homogenised heart tissues comprised three subsequent supernantants and the pellet remained as an insoluble fraction after each extraction.

SDS-PAGE

The aliquots of the tissue extracts containing 50 μg of protein were fractionated by sodium dodecyl sulphate – polyacrylamide-gel electrophoresis (10% gels) according to Laemmli [8]. Gels were stained with Coomassie Blue R-250. Protein concentration was determined by a BCA protein assay using crystalline bovine serum albumin as a standard [9].

Spectrophotometric analysis

Spectrophotometric analysis was performed with a Perkin–Elmer LS-50B spectrometer. Fluorescence and fluorescence excitation spectra of tissues were recorded *ex vivo* and during various stages of homogenization. Fluorescence reflects the intrinsic features of the tissue, which are obtained in the form of fluorescence spectra. Fluorescence excitation spectra at some approximation represent the absorption of tissue. During fluorescence measurements, the excitation wavelength (λ_{ex}) is kept constant, and the detector scans the selected wavelength

region thus obtaining fluorescence spectra, while measuring fluorescence excitation the detection wavelength (λ_{fl}) is kept constant and the excitation wavelength scans the selected region.

Analyses of liquid samples were performed using a 1 cm thick quartz quvette. Small unstained SDS-gel sections containing an appropriate group of proteins were cut out from gels, fixed on quartz slides and used for spectroscopic investigations in a reflection mode. The excitation and detection slits in all measurements were 5 nm and 7 nm, respectively.

RESULTS AND DISCUSSION

Our previous studies on HB and MC tissue autofluorescence have revealed that the intensity of HB fluorescence in the visible spectral range (430–550 nm) is about two times higher in comparison with that of MC (exciting in 330–380 nm spectral range) [10]. On the basis of spectral data, experiments of HCS visualization *ex vivo* were performed [11, 12].

In this study, MC, HB and CT tissue extracts were fractionated by SDS-PAGE and the gels were investigated by spectroscopic methods with the aim of determining the localization of endogenous fluorophores in the fractions of these extracts. It was of interest, because HB protein composition was investigated for the first time, though MC composition is widely explored [13–15].

Heart tissues were disrupted and homogenized in several stages. The choice of composition for extraction buffers (see Materials and Methods) was determined by the requirements of extensive solubilization procedure and the extracts' compatibility for the further spectrophotometric studies. Although treatment with nonionic detergents proved to be a very useful tool for the solubilization of proteins [16], extracts containing Triton X-100 and/or Nonidet-40 showed a strong UV absorbance, thus making spectrophotometric measurements difficult. However, even after treatment with high ionic strength buffers and with high levels of dissociating agents such as urea and SDS, some differences in fluorescence spectra of the remaining pellets from different tissues were observed (results not shown).

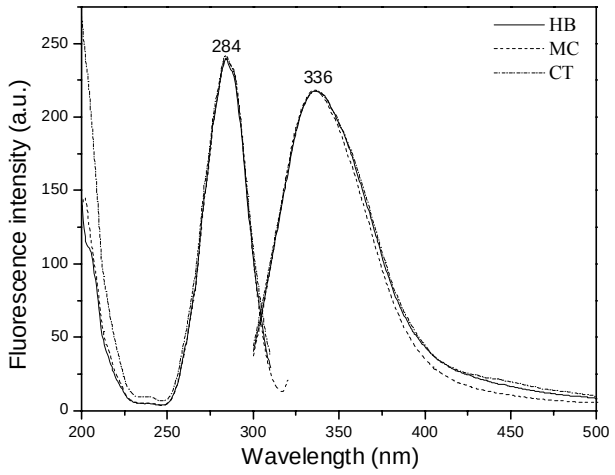

Fig. 2. Fluorescence excitation spectra (λ_{fl} = 335) nm and fluorescence spectra (λ_{ex} = 280 nm) of HB, MC and CT homogenates (first soluble fraction) normalized at 284 nm and 336 nm, respectively

Figure 2 represents spectra of the first soluble fraction of heart tissues (fluorescence and fluorescence excitation spectra of all soluble fractions were quite similar). To compare the spectra in their form, the obtained spectra were normalized. Normalization of two spectra is performed by multiplying the less intense one by a certain number that maximums of both spectra have the same intensity value. A characteristic band around 284 nm was detected in the excitation spectra and a wide band around 336 nm in the fluorescence spectra. This pair of excitation and emission bands could be assigned to tryptophan (W) residues. Consequently, fluorescence of the soluble fractions is excitable only in the region of the absorption of aromatic amino acids. By exciting in the UVA range no fluorescence in the blue region was observed as in tissue samples from myocardium and

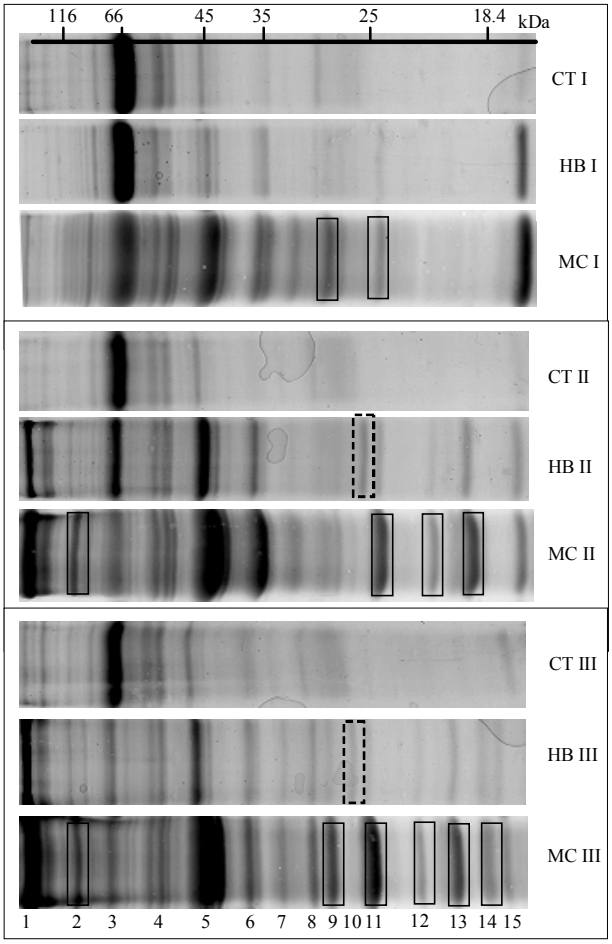

Fig. 3. SDS-PAGE of heart tissue extracts. On the top – mass scale, at the bottom – numbers of protein groups. Numbers I, II, III represent the first, the second and the third fractions respectively. Groups of proteins marked by a solid line are specific to MC, and groups specific to HB are marked by a dashed line

Table. Proteins present in soluble fractions of heart tissues studied by spectroscopic methods

Group of proteins (No.)	Apparent molecular weight, kDa	Soluble fractions of CT			Soluble fractions of HB			Soluble fractions of MC		
		I	II	III	I	II	III	I	II	III
A (1)	~160–155					++	++		++	+++
B (3)	~66	+++	+++	+++	+++	++	+	++	+	+
C (4)	~57–52	+	+	+	+	+	+	++	++	++
D (5)	~45	+	+	+	+	++	++	++	+++	+++

heart conduction system [10]. Thus, fluorophores responsible for emission in this spectral region precipitated out as an insoluble fraction, which was proved by spectroscopic data (results not shown).

SDS-PAGE electrophoregrams of soluble fractions of heart tissues were composed of multiple bands of proteins (Fig. 3). Though electrophoresis results slightly varied depending on the tissue specimen itself and how fine it was mashed, some regularity was observed. We distinguished 15 main protein groups according to differences in quantity between particular heart tissues. During the subsequent studies proteins of the same weight were characterized according to fluorescence and fluorescence excitation spectra. To this end, four the most apparent groups, noted as A, B, C and D, were chosen (summarized in Table).

The proteins (~160–155 kDa in weight) assigned to group A appeared only in soluble fractions II and III of HB and MC. CT extracts contained negligible quantities of these proteins in soluble fractions. Therefore these proteins seem to be specific for muscular tissues (MC and HB) and could be considered for differentiation between muscular and connective tissues.

This group of proteins had very similar fluorescence and fluorescence excitation spectra as the protein group D (around 45 kDa). As is seen from Fig. 4 (160–155 kDa

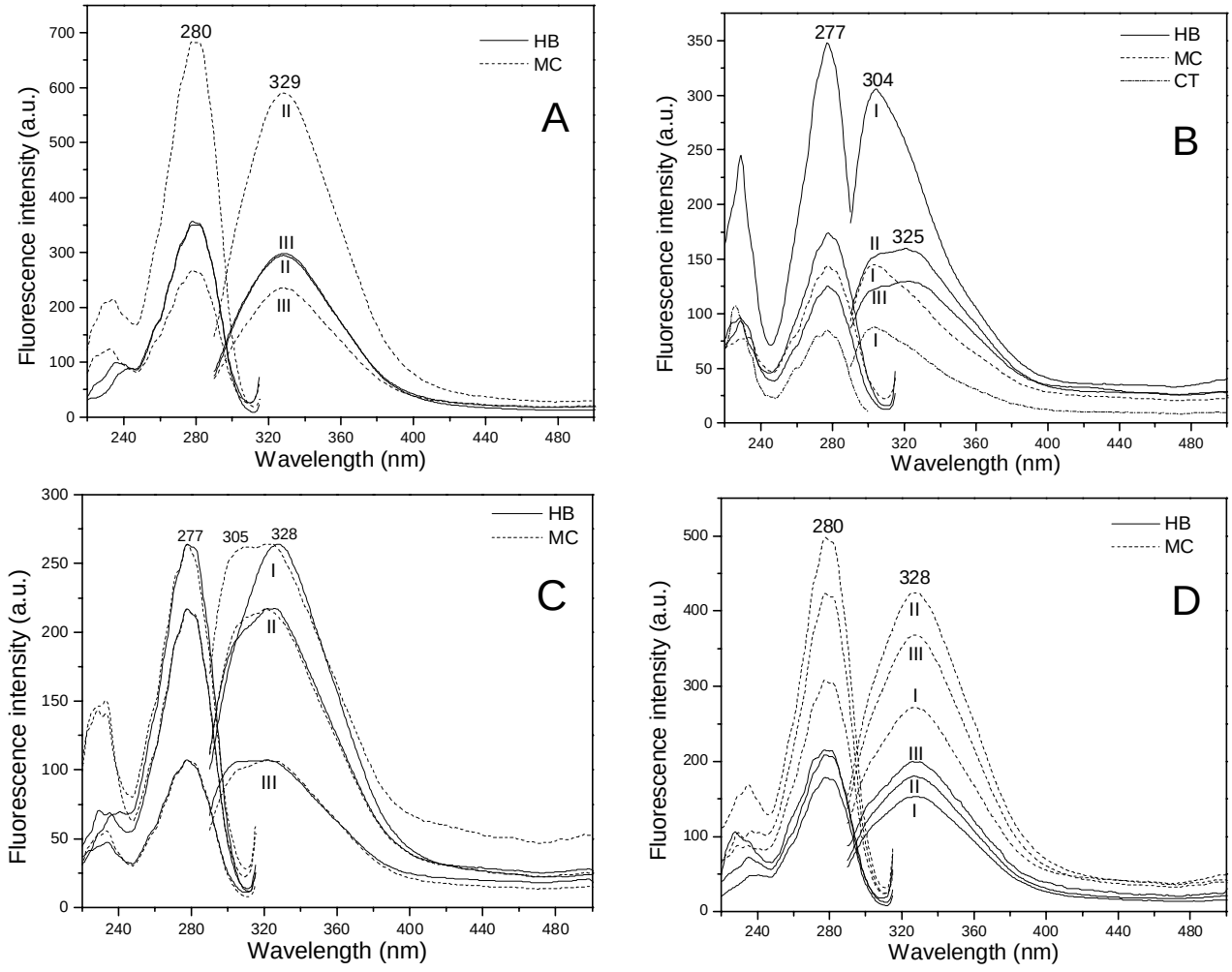

Fig. 4. Fluorescence excitation spectra (λ_{fl} = 329 nm) and fluorescence spectra (λ_{ex} = 270 nm) of HB, MC and CT soluble fractions (I, II, III); groups of proteins: (A, 160–155 kDa) (A), (B, 66 kDa) (B), (C, 57–52 kDa) (C) and (D, 45 kDa) (D)

proteins (A) and 45 kDa proteins (D)), the excitation spectra had a typical band around 280 nm and a wide band around 328 nm in the fluorescence spectra. This pair of bands again can be attributed to W residue, the dominant fluorophore in proteins. The emission maximum of W in neutral solvent is around 310 nm, in water it occurs near 350 nm and is highly dependent upon polarity and the surrounding environment because of the presence of two overlapping electronic states [17]. Fluorescence bands observed around 328 nm indicated that W residues in this case were localized inside the protein and shielded from solvent by the protein matrix. 270 nm was chosen for fluorescence excitation, because at this wavelength both fluorescent amino acids – tyrosine (Y) and W – are excited.

The protein group B (~66 kDa in weight) dominated in all fractions of CT and in fraction I of HB. In fractions II and III of HB it appeared in smaller quantities. In MC this strip appeared in all three fractions but was even less intensive than in HB. Interestingly, ~66 kDa proteins present in HB and CT soluble fractions were found as being strongly glycosylated while MC peptides were not (results not shown). Thus, it can be assumed that proteins of heart tissues having very close molecular weights are not identical. The spectra of this protein group were measured from fraction I of all tissues and from fractions II and III of HB (Fig. 4B). The results showed that fluorescence and fluorescence excitation spectra of fraction I were very similar for all tissues and had a fluorescence band at 304 nm, which can be assigned to Y residue emission. Generally, the emission of proteins is determined mainly by W which absorbs at the longest wavelengths and possesses the largest extinction coefficient. Also, because of such W absorption, energy absorbed by Y residues is often transferred to W residues in the same protein and the fluorescence of Y residues is strongly quenched. The fluorescence of Y residues could be observed only in proteins where W is absent or is located far from Y and there is no energy transfer (Forster's radius for the W–Y pair is 9 – 18 Å) [17]. The obtained results indicate that W is almost absent in fraction I proteins. A broad fluorescence band consisting of two overlapping bands around 304 nm and 325 nm is observed in the fluorescence spectra of fractions II and III of HB (Fig. 4B). Fluorescence around these wavelengths can be attributed to Y and W residues. Therefore these proteins are different from those observed in fraction I. These protein bands in fractions II and III are composed of proteins containing both amino acids – Y and W.

The fluorescence spectra of 57–52 kDa proteins (group C, Fig. 4C) were obtained from fractions I, II and III of HB and MC tissues. Spectra of each fraction were normalized for clarity. The main spectral differences were observed between samples of fraction I. A fluorescence band with a maximum around 328 nm was detected in HB proteins, indicating the presence of W in these proteins. The fluorescence spectra of proteins in MC were composed of two overlapping bands with the maxima around 305 nm and 325 nm, implying that this protein group contains additional proteins in which W is absent or is far from Y. Fluorescence spectra of fractions II and III of both HB and MC were composed of two overlapping bands – around 305 nm and 325 nm, implying a similar composition of the proteins.

Several protein groups (2, 9, 11, 12, 13 and 14) have been detected exceptionally in the second and in the third fractions of MC as seen from SDS-PAGE data (Fig. 3). These proteins seem to be specific to MC, which could be distinguished from other tissues by staining one of these proteins.

It is of interest to note that the protein group (10, ~26 kDa in weight) appeared only in fractions II and III of HB. At present, we have no additional information on the identity of these peptides. However, this might be a protein or a group of proteins, which is specific only to HB. Labelling this protein with a specific dye or a fluorescing agent, the conduction system of the heart could be marked and easily visualized under appropriate illumination conditions.

CONCLUSIONS

In this work, protein composition of the conduction system of the heart was investigated for the first time. Investigations were made by electrophoresis and spectroscopic methods. The differences in protein composition among the heart tissues (CT, HB and MC) were observed. According to electrophoresis data, one protein group has been found specific to both muscular tissues. Several protein groups were specific only to MC, while one group (around 26 kDa) was distinguished as characteristic mainly to HB. Fluorescence of the proteins of the soluble fraction was mainly determined by emission of W and Y residues, while fluorophores responsible for the fluorescence in the visible region remained in the insoluble fraction. Spectroscopic measurements of the most apparent groups in different heart tissues indicated that some proteins of the same weight exhibit different emission spectra. Therefore, fluorescence spectroscopy combined with electrophoresis could be useful for a detailed protein identification and distinguishing of tissues.

Received 24 January 2006
Accepted 23 June 2006

References

1. Development of the Cardiac Conduction System. Novartis Foundation. John Wiley and Sons Ltd. Chichester UK, 2003.
2. Langman J, Woerdeman MW. Atlas of Medical Anatomy. Philadelphia: Saunders Press, 1982.
3. Perk M, Flynn GJ, Gulamhusein S et al. PACE 1993; 16: 1701-12.
4. Kochiadakis GE, Chrysostomakis SI, Kalebubas MD et al. Chest 2001; 120: 233–9.

5. Nilsson AMK, Heinrich D, Olajos J et al. Spectrochimica Acta 1997; Part A: 53.
6. Синев АР, Крымский ИД. Хирургическая анатомия проводящей системы сердца. Медицина, 1985.
7. Cardiovascular Pathology. Ed. Silver MD. New York: Churchill Livingstone, 1991.
8. Laemmli UK. Nature 1970; 227: 680–5.
9. Smith PK, Krohn RI, Hermanson GT et al. Anal Biochem 1985; 150(1): 76–85.
10. Žurauskas E, Bagdonas S, Bandzaitytė L et al. Lithuanian Journal of Physics 2004; 44: 35–40.
11. Venius J, Žurauskas E, Bagdonas S et al. Biomedical Engineering Proceedings of International Conference, Kaunas 2004: 221–6.
12. Venius J, Žurauskas E, Bagdonas S et al. Acta Bio-Optica et Informatica Medica 2005; 12: 6–9.
13. Otto A, Thiede B, Müller E-C et al. Electrophoresis 1996; 17: 1643–50.
14. Jager D, Jungblut PR, Muller-Werdan U. J Chromatogr Part B 2002; 771(1–2): 131–53.
15. Muller EC, Thiede B, Zimny-Arndt U et al. Electrophoresis 1996; 17(11): 1700–12.
16. Scopes RK. Protein Purification: Principles and Practice. 3rd ed., New York: Springer; 1993.
17. Lakowicz JR. Principles of Fluorescence Spectroscopy. New York, 1998.

J. Venius, D. Labeikytė, E. Žurauskas, V. Strazdaitė, S. Bagdonas, R. Rotomskis

ŠIRDIES AUDINIŲ EKSTRAKTŲ TYRIMAS SPEKTROSKOPIJOS METODAIS

Santrauka

Širdies laidžioji sistema (ŠLS) yra raumeninio tipo audinys, generuojantis ir perduodantis bioelektrinius impulsus. Chirurginių operacijų metu galimas ŠLS pažeidimas, nes jos audinys yra sunkiai atskiriamas nuo darbinio širdies raumens – miokardo (MK). Šio darbo tikslas buvo ištirti MK ir ŠLS dalies – Hiso pluošto (HP) – audinių baltyminės sudėties skirtumus, kurie padėtų nustatyti fluoroforų pasiskirstymą šiuose audiniuose, o tai savo ruožtu sudarytų prielaidas optinės-fluorescencinės biopsijos metodu gaunamam ŠLS ir MK vaizdui. ŠLS ir MK homogenatų spektroskopiniai tyrimai rodo, kad tirpių frakcijų fluorescencijos spektruose vyrauja triptofano (W) ir tirozino (Y) fluorescencija, tuo tarpu regimojoje spektro srityje fluorescuojantys fluoroforai sunkiai ekstrahuojami iš audinių mėginių ir pasilieka netirpiose frakcijose. Išanalizavus elektroforegramas buvo aptikta keletas baltymų grupių, būdingų tik MK ar HP. Elektroforeze išskirtos tam tikros charakteringos baltymų grupės buvo tirtos spektroskopijos metodais. Tyrimai rodo, kad kai kurie tos pačios masės baltymai, išskirti iš skirtingų audinių, pasižymi skirtingais fluorescencijos spektrais.