

# ELEKTROFORETIČKA ANALIZA DENTINSKOG EKSTRACELULARNOG Matriksa (KISELIH FOSFOPROTEINA, PROTEOGLIKANA I GAMAKARBOKSIGLUTAMAT PROTEINA)

## ELECTROPHORETIC ANALYSIS OF DENTIN EXTRA CELLULAR MATRIX (ACID PHOSPHOPROTEINS, PROTEOGLYCAN AND GAMMA-CARBOXY-GLUTAMATE OF PROTEINS)

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### Kratak sadržaj

Dentin, kao specifično tvrdo zubno tkivo, bogato je organskim materijama koje čine oko 20% njegove težine i 33% zapremine. Bazična organska komponenta dentina je kolagen. Međutim, nekolageni organski sastav dentina nedovoljno je proučen. Dentin i njegova složena ultrastruktura, molekularni satav i biohemski procesi koji se u ovom tkivu odigravaju još uvek su nedovoljno razjašnjeni, pa samim tim predstavljaju i izazov za istraživače.

Autori su postavili cilj da, primenom savremene elektroforetičke analize frakciju dentina, analiziraju sastav ekstracelularnog matriksa dentina i dobijene rezultate dovedu u korelaciju sa podacima iz literature.

Izdvajanje proteinâ u dentinu izvedeno je tehnikom natrijum dodecil sulfatne poliakrilamidne gel elektroforeze (SDS PAGE elektroforeze) kojom se vrši separacija polipeptida na osnovu razlike u njihovim molekularnim masama. Elektroforetična analiza proteina je veoma značajna u bazičnim istraživanjima grupa (familija) proteina koji čine organsku osnovu dentinskog tkiva.

Rezultati SDS poliakrilamidne gel elektroforeze frakcija dentina pokazuju veliku koncentraciju proteina i proteinskih familija. Sa obzirom na veliku koncentraciju proteina kao i rasprostranjenost u većem broju frakcija dentina na mestima različitih molekularnih masa, izvodi se zaključak da se radi o porodici fosfoproteina čija se masa kreće u intervalu od 35 do 158 kDa. SDS PAGE analiza tri uzorka sa visokim koncentracijama frakcije broj 20 dentina potvrđuje prisustvo proteoglikana u nekolagenom sastavu dentina. Njihova masa je oko 75 kDa. Takođe je dokazano i prisustvo gama karboksigtamat proteina (Gla proteini), čija karboksilna grupa omogućava njihov afinitet prema jonima kalcijuma.

**Ključne reči:** SDS PAGE elektroforeza, fosfoproteini, proteoglikani, Gla proteini, mineralizacija dentina

### Abstract

Being a specific hard dental tissue, dentin is rich in organic matter which comprises about 20% of its weight and 33% of its volume. Basic organic component of dentin is collagen. However, non-collagen organic structure of dentin has not been sufficiently studied. Dentin and its compound ultra-structure, molecular structure and biochemical processes within this tissue have not been sufficiently cleared out yet and as such they represent a challenge for researchers.

The authors set themselves a goal to analyse the structure of dentin extra cellular matrix by applying contemporary electrophoretic analysis of dentin fraction, and to bring those results into correlation with literary data.

Extracting proteins within dentin was performed by using a technique of sodium dodecyl sulphate poly acryl amide elecrophoresis gel (SDS PAGE electrophoresis) which separates polypeptides according to the difference in their molecular masses. Electrophoretic analysis of proteins is crucial in basic researches of the groups (families) of proteins which compose organic base of dentin tissue.

The results of SDS poly acryl amide electrophoresis gel fractions of dentin indicate a great concentration of proteins and protein families. Due to big concentration of proteins as well as due to their being widely spread in greater number of dentin fractions in places of different molecular masses, it can be concluded that we are dealing with phosphoprotein family whose mass moves in intervals of 35 to 158 kDa. SDS PAGE analysis of three samples with high fraction concentrations of dentin number 20 confirms the presence of proteoglycan in non-collagen dentin structure. Their mass is about 75 kDa. The presence of gamma-carboxy-glutamate proteins (Gla proteins) has also been proven and their carboxyl group enables their affinity towards calcium ions.

**Key words:** SDS PAGE electrophoresis, phosphoproteins, proteoglycans, Gla proteins, dentin mineralisation

## ***Uvod***

Najveći deo organskog matriksa dentina čini kolagen (oko 90%), dok ostatak čine nekolageni proteini i proteoglikani sa dominantnim anionskim karakterom, kao i izvesne lipidne komponente.<sup>1</sup>

Pored kolagena, nekolageni makromolekuli predstavljaju vrlo važan konstituent organskog matriksa dentina. Ovi makromolekuli čine ekstracelularnu masu mineralizovanog tkiva i mogu se klasifikovati u sedam kategorija – fosfoproteini, proteoglikani, gama-karboksiglutamat (Gla) proteini, kiseli glikoproteini, faktori rasta, lipidi i serumski proteini. Grupa fosfoproteina je najbrojnija. Osnovni makromolekul ove grupe je fosforin, visoko fosforilisani fosfoprotein jedinstven za dentin, koji čini oko 50% nekolagenog proteinskog sastava dentina. Molekularna masa porodice fosfoproteina se nalazi u intervalu 35 do 158 kDa kod čoveka (30 do 100 kDa za fosfoproteine locirane u dentinu eksperimentalnih miševa).<sup>2</sup> Fosforin se nalazi samo u mineralnom matriksu i nije pronađen u predentinu<sup>3</sup> a produkt je visoko diferentovanih odontoblasta, on se transportuje kroz odontoblastni produžetak i deponuje se na mineralizacionom frontu.<sup>4</sup>

Mali proteoglikani, glikozaminoglikani koji sadrže dermatin, hondroitin i keratin sulfat, takođe su otkriveni u dentinu. U dentinu se još mogu naći i dekorin, mali proteoglikan, često udružen sa kolagenim vlaknima, kao i biglykan, proteoglikan sa dva glikozaminoglikanska lana. Proteoglikani locirani u predentinu su veći od onih u dentinu. Njihova molekularna masa je oko 75 kDa.<sup>5</sup>

Gla proteini (gama karboksiglutamat proteini) nose ovaj naziv jer sadrže u svom sastavu jedinstvenu amino-kiselinu, gama-karboksil glutamsku kiselinu. Reakcija karboksilacije zavisi od prisustva vitamina K.<sup>6</sup> Karboksilne grupe potvrđuju sposobnost Gla proteina da vezuju kalcijum.<sup>7</sup> Postoje dve grupe Gla proteina u kostima i dentinu. To su Gla proteini tipa osteocalcina i matriksni Gla protein (MGP).<sup>8</sup>

Matriksni Gla protein ima molekularnu masu od 10,6 kDa, i predstavlja jednu od organskih komponenti matriksa kosti i dentina. Nivo Gla proteina u dentinu je skoro isti kao i nivo ovih proteina u koštanom tkivu. Literaturni podaci ukazuju da postoji veza između 1,25-dihid-

## ***Introduction***

The greater part of dentin organic matrix is comprised of collagen (about 90%), whereas the rest of it is comprised of non-collagen proteins and proteoglycans with dominant anion character as well as of certain lipid components.<sup>1</sup>

Beside collagen, non-collagen macromolecules represent a very important constituent of dentin organic matrix. These macromolecules comprise extra cellular mass of mineralized tissue and they can be classified in seven categories – phosphoproteins, proteoglycans, gamma-carboxy-glutamate (Gla) proteins, acid glyco-proteins, growth factors, lipids and serum proteins. Phosphoprotein group is the most numerous. The basic macromolecule of this group is phosphorin, highly phosphorylated phosphoprotein unique for dentin, which comprises about 50% of non-collagen protein structure of dentin. Molecular mass of phosphoprotein family is within the interval of 35 to 158 kDa in a human (30 to 100 kDa for phosphoproteins located in dentin of experimental mice).<sup>2</sup> Phosphorine is situated only in mineral matrix and it is not found in predentin<sup>3</sup> and it is the product of highly differentiated odontoblasts. It is transported through odontoblastic extension and sent to mineralization front.<sup>4</sup>

Small proteoglycans, glycosaminoglycans which contain dermatine, chondroitin and keratin sulphate are also found in dentin. Within dentin we can also find decorin, small proteoglycan, often coupled with collagen fibres, as well as biglycan, proteoglycan with two glycosaminoglycan chains. Proteoglycans found in predentin are larger than those in dentin. Their molecular mass is about 75 kDa.<sup>5</sup>

Gla proteins (gamma-carboxy-glutamate proteins) are called thus because they contain unique amino-acid, gamma-carboxyl glutamate acid. Carboxylation reaction depends on the presence of vitamin K.<sup>6</sup> Carboxyl groups confirm ability of Gla proteins to bind Calcium.<sup>7</sup> There are two groups of Gla proteins in dentin and bone tissue. Those are Gla proteins of osteocalcine type and matrix Gla protein (MGP).<sup>8</sup>

Matrix Gla protein has molecular mass of 10,6 kDa, and it represents one of the organic components of bone and dentin matrix. The level of Gla proteins in dentin is almost the same as the level of these proteins in bone tis-

doksi-vitamina D3 i Gla proteina. (1,25-dihidroksi vitamin D3 stimuliše sintezu Gla proteina).<sup>9</sup> Osim u dentinu i kosti, osteokalcin se nalazi i u serumu.<sup>9,10</sup>

sue. Literary data show that there is a connection between 1,25-dihydroxy-vitamins D3 and Gla proteins (1,25 – dihydroxy-vitamins D3 stimulates Gla protein synthesis).<sup>9</sup> Except in dentin and bones, osteocalcine is found in serum as well.<sup>9,10</sup>

## **Cilj istraživanja**

Cilj ovog istraživanja je da se izvođenjem savremene elektroforetičke analize frakcija dentina analizira sastav ekstracelularnog matriksa dentina i dobijeni rezultati dovedu u korelaciju sa podacima iz literature.

## **The research objective**

The objective of this reasearch is to analyse the structure of dentin extra cellular matrix by performing contemporary electrophoretic analysis of dentin fractions and to bring these results into correlation with literary data.

## **Materijal i metode istraživanja**

### **Preparacija uzorka**

Zubi u masi od 100 grama su zamrznuti u tečnom azotu i razbijeni na veoma male delove hidrauličnom presom do konzistencije praha. Zamrzavanju zuba prethodilo je precizno odvajanje gleđi i cementa mehaničkim uklanjanjem slojeva tkiva do dentina. Smrznuta masa zuba u prahu je tretirana sa gvanidin hidrohloridom u 50 mM natrijum acetatu, pH vrednosti 5.8. Cilj ovog postupka je uklanjanje ćelijskih ostataka i makromolekula koji ne potiču iz dentina.<sup>11,12</sup>

SDS PAGE elektroforeza se izvodi u poliakrilamidnim gelovima koji sadrže natrijum dodecil sulfat. Gelovi za ovu elektroforezu se pripremaju u laboratoriji neposredno pred početak eksperimenta. Poliakrilamidni gelovi su polimerizovani rastvori akrilamida i bis-akrilamida. Sam akrilamid formira linearne polimere. Bis-akrilamid, sa svoje strane, omogućava unakrsno povezivanje između poliakrilamidnih lanaca. Tako se polimerizuje gel koji ima ulogu sita, pri čemu su veće pore pri vrhu gela, dok se veličina pora postepeno smanjuje kako se približava donja ivica gela.<sup>13,14,15</sup>

Tokom SDS PAGE elektroforeze, svi proteini u uzorcima koji se na početku procesa nalaze u inertnom gelu za postavljanje uzorka (stacking gel), na vrhu separacionog, poliakrilamidnog gela (running gel), migriraju prema anodi (negativnoj elektrodi) na dnu gela.<sup>16</sup>

U toku PAGE, nivo migracije SDS-tretiranih proteina je determinisan molekularnom

## **The research material and its methods**

### **Sample preparation**

Teeth of 100 grams in volume are frozen in the liquid azoth and smashed into very small pieces with hydraulic press into consistent powder. Prior to teeth freezing, a precise separation of glaze and cement is done by mechanically removing tissue layers from dentin. Frozen powdered teeth mass is treated with guanidine hydrochloride in 50 mM sodium acetate, with the pH value of 5.8. The objective of this procedure is to remove the cell remains and macromolecules which do not originate from dentin.<sup>11,12</sup>

SDS PAGE electrophoresis is done in poly acryl amide gels which contain sodium dodecyl sulphate. Gels needed for this electrophoresis are prepared in laboratories immediately before the beginning of the experiment. Poly acryl amide gels are polymeric solutions of acryl amides and bis-acryl amides. Acryl amide itself forms linear polymers. Bis-acryl amide, on the other hand, enables cross-connection among poly acryl amide chains. This is the way to polymerize a gel acting as a sieve with larger pores at the top which become smaller as lower edge of the gel is being approached.<sup>13,14,15</sup>

During electrophoresis SDS PAGE, all the proteins in the samples which are, at the beginning of the process, situated inside the sample-setting inert gel (stacking gel), on the top of the separational, poly acryl amide gel (running gel), they migrate towards the anode (negative electrode) at the bottom of the gel.<sup>16</sup>

During PAGE, the level of SDS-treated proteins migration is determined by molecular pro-

masom proteina. Proteini prolaze kroz "rešeto" gela pri čemu proteini sa većom masom ostaju zarobljeni u višim delovima gela, a manji prodiru naniže, gde je i dijametar pora sve manji.

### Vizuelizacija uzoraka

Protokol za bojenje (Coomassie Blue Staining Protocol) pri vizuelizaciji proteina u SDS PAGE-u podrazumeva uranjanje gela u rastvor sa 50% etanola i 10% sirčetne kiseline na vremenski period od najmanje jednog sata.<sup>17,18</sup>

Nakon toga sledi ispiranje gela u vodi i njegovo razvijanje u 0.04%-nom formalinu (35% formaldehida u vodi) i 2%-nom natrijum karbonatu pri intenzivnom mešanju gela. Tretirani gelovi, sada obojeni Silver Staining tehnikom, čuvaju se na temperaturi od 4°C u 1%-nom rastvoru sirčetne kiseline, sve do trenutka kada se vrši analiza rezultata.<sup>19</sup>

Po završetku vizuelizacije dobijenih rezultata elektroforeze biraju se tačke na gelu (proteini se nakon bojenja uočavaju kao tamne mrlje) koje se isecaju iz gela i upućuju na masspektrometrijsku analizu.

Dobijeni rezultati masspektrometrijskih analiza u vidu determinisanih sekvenci amino-kiselina se zatim upoređuju sa dostupnim bazama podataka u kojima se nalaze sekvene do sada otkrivenih i opisanih proteina. Analiza sličnosti sekvenci amino-kiselina omogućena je korišćenjem specijalnih baza podataka i kojima se nalaze svi do sada otkriveni lanci amino-kiselina koji ulaze u sastav polipeptida.

### Rezultati istraživanja

Rezultati SDS poliakrilamidne gel elektroforeze frakcija dentina (DE 52, 11-26) pokazuju veliku koncentraciju proteina i proteinских familiјa, posebno u frakcijama (slika 1).<sup>11, 12, 13, 14, 15, 16</sup> Sa

tein mass. While going through the gel "sieve", proteins with larger mass stay trapped in the higher parts of the gel whereas the ones with smaller mass continue to penetrate lower in the gel where pore diameter becomes smaller.

### Sample Visualisation

Colouring protocol (Coomassie Blue Staining Protocol) during protein visualisation in SDS PAGE entails plunging the gel into a solution of 50% ethanol and 10% octane acid within the time framework of at least one hour.<sup>17,18</sup>

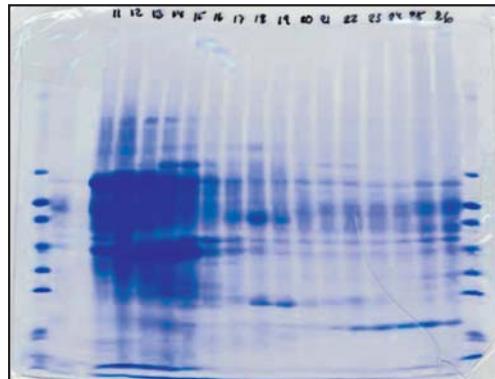
After that, the gel is plunged into water and then developed on a 0,04% formalin (35% formaldehyde in water) and 2% sodium carbonate during intensive gel mixing. Treated gels, coloured by Silver Staining technique, are being preserved at 4°C temperature in a 1% solution of octane acid up to the moment when results analysis is being done.<sup>19</sup>

As soon as the visualisation of the achieved electrophoresis results is finished, the points on the gel are chosen (after colouring, proteins can be seen as dark marks) which are then cut from the gel and sent to massspectrometric analysis.

Achieved massspectrometric analysis results in the shape of determinate sequences of amino-acids are then compared to the available data base in which the sequences of the discovered and described proteins are found. The analysis of the amino acid similarities is enabled by the use of special data base within which there are all the discovered amino acid chains which comprise polypeptides.

### The research results

The results of the SDS poly acryl amide gel electrophoresis of dentin fractions (DE 52, 11-26) show a big concentration of proteins and protein families, especially in fractions (Figure 1)<sup>11,12,13,14,15,16</sup>. Due



Slika 1. SDS PAGE elektroforeza frakcija dentina 11-26, DE 52

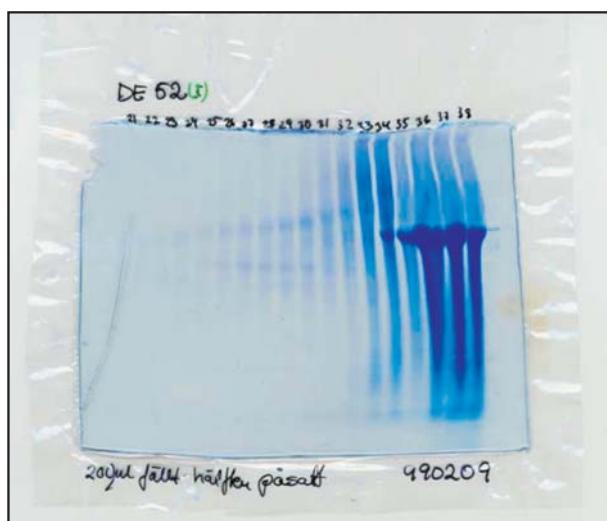
Figure 1. SDS PAGE electrophoresis of dentin fraction 11-26, DE 52

obzirom na veliku koncentraciju proteina kao i rasprostranjenost u većem broju frakcija dentina na mestima različitih molekularnih masa, izvodi se zaključak da se radi o porodici fosfoproteina čija se masa kreće u intervalu od 35 do 158 kDa. Takođe se može pretpostaviti značajnije prisustvo kiselih glikoproteina procenjene molekularne mase između 60 i 95 kDa. Neke od ovih frakcija su posebno tretirane elektroforetskim metodama u cilju dobijanja egzaktnih rezultata.

Gruba analiza većeg broja frakcija dentina (21-38) otkriva postojanje visoko fosforilisanih, kiselih fosfoproteina i kiselih glikoproteina u višim frakcijama dentina (slika 2).

to a big concentration of proteins as well as due to their being widely spread in larger number of dentin fractions in places of different molecular masses, it can be concluded that we are dealing with phosphoprotein family whose mass moves in intervals of 35 to 158 kDa. A more meaningful presence of acid glyco-proteins of estimated molecular mass between 60 and 95 kDa can also be assumed. Some of these fractions have been separately treated with the aim of achieving exact results.

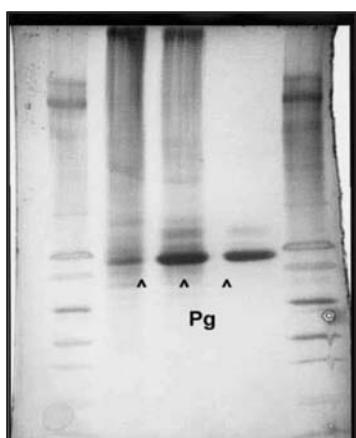
Rough analysis of a larger number of dentin fractions (21-38) reveals the existence of highly phosphorized, acid phosphoproteins and acid glycol-proteins in higher dentin fractions (Figure 2).



Slika 2. SDS PAGE elektroforeza frakcija dentina 21-38, DE 52

Figure 2. SDS PAGE electrophoresis of dentin fraction 21-38, DE 52

SDS PAGE analiza tri uzorka sa visokim koncentracijama frakcije 20 dentina potvrđuje prisustvo proteoglikana u nekolagenom sastavu dentina. Njihova masa je oko 75 kDa. Podatak da su za identifikaciju proteoglikana u uzorku bili potrebni uzorci većih koncentracija dentina, ukazuje da je njihova količina u organskom matriksu mala (slika 3).

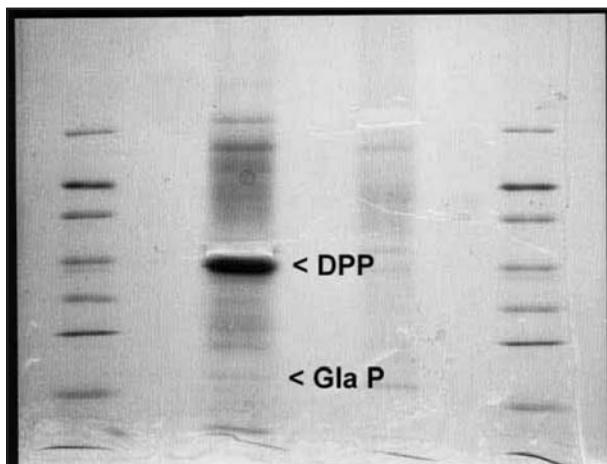


Slika 3. Frakcija dentina 20. (Strelica pokazuje proteoglikane.)

Figure 3. Dentin fraction 20. (Arrows show proteoglycans.)

SDS PAGE analysis of three samples with high fraction concentrations in dentin number 20 confirms the presence of proteoglycan in non-collagen dentin structure. Their mass is about 75 kDa. The fact that samples with higher dentin concentration are needed in order to identify proteoglycan in a sample, points out that their quantity in organic matrix is small (Figure 3).

Analiza rezultata jednodimenzionalne gel elektroforeze dentinskih frakcija 15 i 21 pokazuje prisustvo dentin fosfoproteina (DPP), i Gla proteina izuzetno malih molekularnih masa (slika 4).



Analysis of the results of one-dimensional gel electrophoresis of dentin fractions 15 and 21 shows the presence of dentin phosphoproteins (DPP) and Gla proteins of remarkably small molecular masses (Figure 4).

Slika 4. Frakcije 15 i 21. (Strelice pokazuju visoku koncentraciju dentin fosfoproteina i GLA proteina u malim koncentracijama i sa malim molekularnim masama.)

Figure 4. Fractions 15 and 21. (Arrows show high concentration of dentin phosphoproteins and Gla proteins in noticeably small concentrations and with small molecular masses.)

## Diskusija

Rezultati istraživanja pokazuju visoku koncentraciju fosfoproteina u ekstracelularnom matriksu dentina. Interval molekularnih masa u kojem se ovi proteini mogu naći je od 60 do 120 kDa (slike 1,2,4). Dobijeni podaci su u skladu sa podacima iz literature.<sup>20,21,22</sup> Poznato je da je porodica fosfoproteina najzastupljenija ekstracelularna komponenta organskog matriksa. Prema nekim autorima, DPP čini više od 50% svih nekolagenih proteina ekstracelularnog matriksa dentina.<sup>21</sup> Ovaj podatak je potvrđen i rezultatima istraživanja u radu.

Fosfoproteini predstavljaju grupu fosforilisanih dentin proteina. U literaturi su poznati i kao DPP (dentin fosfoprotein, najistaknutije ime za protein, tj. za porodicu kiselih proteina jedinstvenu za dentin), PP-H i fosfoforini. DPP je najkiseliji poznati protein sa pI vrednošću od 1.1, zahvaljujući visokom sadržaju fosfata i karboksilnih grupa.<sup>23</sup> Korenski dentin ima upola manju količinu fosfoproteina u poređenju sa kruničnim dentinom. Funkcionalni značaj ove razlike nije poznat.<sup>21</sup>

Najzastupljeniji protein ove grupe je dentin fosfoprotein (DPP), visoko fosforilisani fosfoprotein, po nekim autorima poznat i kao fosfoforin. DPP je izolovan jedino u dentinskom tkivu pa predstavlja važan faktor diferencijacije

## Discussion

The research results show high concentration of phosphoproteins in extra cellular dentin matrix. The interval of molecular masses in which these proteins have been obtained is from 60 to 120 kDa (Figures 1, 2, 4). Obtained data are in accordance with literary data.<sup>20,21,22</sup> It is known that phosphoprotein family is the most present extra cellular component of organic matrix. According to some authors, DPP comprises more than 50% of all non-collagen proteins of extra cellular dentin matrix.<sup>21</sup> This was also confirmed by the research results during the work.

Dentin phosphoprotein (DPP) is the sourest known protein with pI value of 1.1, thanks to the high concentration of phosphate and carboxylic groups.<sup>23</sup> Root dentin has half as much phosphoproteins as crown dentin does. Functional importance of this difference is not known.<sup>21</sup>

This protein is isolated only within dentin tissue and it represents an important factor in differentiating bone and dentin tissue.<sup>2</sup> It is known that DPP has big affinity towards calcium ions.<sup>21</sup> This obvious ability to bind with calcium points out that this protein has a certain role in dentin mineralization.<sup>2</sup>

Phosphoproteins are characterized by high concentration of phosphoserine (45-50%) and aspartamic acid (35-38%). Their molecular

koštanog od dentinskog tkiva.<sup>2</sup> Poznato je da DPP ima veliki afinitet prema jonima kalcijuma.<sup>21</sup> Ova izrazita sposobnost vezivanja za kalcijum sugerije da ovaj protein ima izvesnu ulogu u mineralizaciji dentina.<sup>2</sup>

Fosfoproteini se karakterišu visokim sadržajem (45-50%) fosfoserina i aspartanske kiseline (35-38%). Njihova molekularna masa je različita kod različitih vrsta.<sup>24</sup> Kod teladi ona iznosi 155kDa, miševa 72 kDa, pacova 90-95 kDa (MacDougall i sar., 1994). Humani dentin sadrži fosfoproteine molekularne mase od 80 do 123 kDa.<sup>25,26</sup> Uprkos razlikama u veličini, izuzetna sličnost u sastavu ovih proteina kod sisara sugerije njihov značaj u formiranju dentina.<sup>27</sup> Razlike u strukturi DPP-a kod pojedinih vrsta neki naučnici objašnjavaju nedostacima tehnika purifikacije proteina.<sup>28</sup> Dentin fosfoprotein (DPP) vezuje velike količine kalcijuma sa relativno visokim afinitetom.<sup>29</sup> Ovaj protein formira nerastvorljive komplekse u prisustvu magnezijuma i kalcijuma.<sup>30,31</sup> Neobični i specifični afinitet prema kalcijumu je u relaciji sa njegovom biološkom ulogom u procesu mineralizacije tkiva. Posmatrajući tecijarnu strukturu proteina DPP uočava se beta formacija u prisustvu jona kalcijuma. Ova struktura ima tendenciju stvaranja negativnog nailektrisanja na površini molekula, pa je moguće očekivati da ta površina stupi u interakciju sa kalcijumom u toku rasta kristala apatita.<sup>32,33</sup>

Rezultati nekih istraživanja ukazuju da se fosfoproteini sintetišu od strane odontoblasta. Ovi proteini veoma brzo stižu do fronta mineralizacije, verovatno zbog njihove sekrecije kroz odontoblastni produžetak. Autoradiografskim istraživanjima se došlo do podatka da se fosforilisani dentin proteini mogu detektovati na dentinskoj strani mineralizacionog fronta u periodu od 1 do 4 sata. Alternativna mogućnost za objašnjenje brzog prisustva fosfoproteina u mineralizacionom frontu jeste da se sintetisani proteini u odontoblastima sekretuju na ćelijskoj granici, a zatim rapidno difunduju kroz predentin sve do fronta mineralizacije.<sup>20</sup> Jedinstveno prisustvo fosfoproteina u dentinu je dokaz kvalitativne razlike organskog matriksa dentina i predentina, kao i uloge odontoblastni produžetaka u održavanju ove razlike.<sup>34</sup> Fosfoproteini imaju visok afinitet za vezivanje jona kalcijuma i mogu imati induktivnu ulogu u procesu mineralizacije.<sup>35</sup>

Prisustvo malih gamakarboksigtamat proteina (Gla proteina) tipa osteokalcina je po-

mass is different in various species.<sup>24</sup> In calves, it is 155 kDa, in mice 72 kDa, in rats 90-95 kDa (Mac Dougall and ass., 1994).<sup>25,26</sup> In spite of the differences in size, remarkable similarity in structure of these proteins in mammals shows their importance in dentin formation.<sup>27</sup> Some scientists explain that the differences in DPP structure with certain species appears due to the lack of protein purification technique.<sup>28</sup> Dentin phosphoprotein (DPP) binds big quantities of calcium with relatively high affinity.<sup>29</sup> This protein forms insoluble complexes in the presence of magnesium and calcium.<sup>30,31</sup> Unusual and specific affinity towards calcium has to do with its biological role in tissue mineralization process. Observing a threefold structure of DPP protein, beta formation is seen in the presence of calcium ions. This structure has a tendency to create negative electricity on the molecule surface and, thus, it is possible to expect the surface to start interacting with calcium during the growth of crystal apatite.<sup>32,33</sup>

The results of some researches point to the fact that phosphoproteins are synthesized by odontoblast. These proteins come to the mineralization front very quickly, probably due to their secretion through odontoblastic extension. Through auto-radiographic researches we came to the data that phosphorized dentin proteins can be detected on the dentin side of mineralization front in the period of 1 to 4 hours. Alternative possibility to explain fast presence of phosphoprotein in mineralization front is for synthesized proteins in odontoblast to secret on the cell border and then to rapidly defund through predentin all the way to the mineralization front.<sup>20</sup> Simple presence of phosphoprotein in dentin is a proof of qualitative difference between organic dentin matrix and predentin as well as of the odontoblast extension role in maintaining the difference.<sup>34</sup> Phosphoproteins have high affinity towards binding calcium ions and can have inductive role in the mineralization process.<sup>35</sup>

The presence of small gamma-carboxy-glutamate proteins (Gla proteins) of osteocalcine type is confirmed in this research. The data about their molecular mass obtained by SDS PAGE electrophoresis are in correlation with the known contemporary literary data. Gla protein contains about 50 amino acids. Molecu-

tvrđeno u radu. Podaci o njihovoj molekularnoj masi dobijeni SDS PAGE elektroforezom su u korelaciji sa poznatim podacima iz savremene literature. Gla protein sadrži oko 50 amino-kiselina. Molekularna masa Gla proteina se kreće u intervalu od 6 do 12 kDa.<sup>36</sup> U svom sastavu ovaj protein sadrži gama karboksil glutaminsku kiselinu i jednu disulfidnu vezu.<sup>37</sup>

Osteokalcin je jedan od najprisutnijih proteina u koštanom matriksu. Ovaj protein lociran je i u ekstracelularnom matriksu dentina.<sup>38</sup> Osteokalcin je sintetisan i sekretovan od strane odontoblasta in vivo<sup>39,40</sup> i u in vitro uslovima.<sup>41,42</sup>

Funkcionalni značaj osteokalcina je u skladu sa karakteristikama svih Gla proteina. Osteokalcin čvrsto vezuje hidroksiapatit i inhibitor je formiranja hidroksiapatita in vitro.<sup>20</sup> Ovi podaci upućuju na moguću ulogu osteokalcina kao negativnog regulatora sinteze koštanog tkiva i dentina.<sup>43</sup> Funkcija ovog proteina još uvek nije jasna. MPG je inhibitor mineralizacije jer se sintetiše u visokim koncentracijama u neskeletnim tkivima.<sup>44,45</sup>

Ovi proteini imaju anjonski karakter i sposobnost vezivanja jona kalcijuma, zbog čega se pretpostavlja da i ova proteinska grupa ima značajnu ulogu u mineralizaciji.<sup>46</sup>

Rezultati istraživanja sprovedenih u eksperimentalnom delu rada nedvosmisleno ukazuju na prisustvo proteoglikana u dentinu. Dobijeni rezultati molekularne mase proteoglikana kreću se oko 75 kDa (slika 3), što odgovara podacima iz literature.<sup>47</sup> Dva najznačajnija mala proteoglikana locirana u dentinu su dekorin i biglycan.<sup>48</sup> Podaci iz literature ukazuju da se proteoglikani sekretuju u predentin i na neki način sprečavaju proces njegove mineralizacije.<sup>20</sup> Istraživanja pokazuju da se 25 – 50% nascentnih proteoglikana gubi u procesu sinteze dentina.<sup>49</sup> Steinfort i saradnici<sup>50</sup> su dokazali rapidnu inkorporaciju proteoglikana u dentinu, dok se istovremeno sa procesom dentinogeneze količina proteina u predentinu smanjuje. Studija Hoshia i saradnika<sup>51</sup> pokazuje da se neposredno pre mineralizacije vlakna kolagena spajaju i postaju veća u svom dijametru. Detekcijom putem imunolokalizacije utvrđeno je da se istovremeno sa fuzijom vlakna kolagena smanjuje koncentracija dekorina. Podaci iz ove studije ukazuju da je jedan od važnih momenata u procesu dentinogeneze upravo smanjenje nivoa proteoglikana dekorina, sa ciljem poboljšanja fuzije kolagena i indukcije mineralizacije.

lar mass of Gla proteins moves in the intervals of 6 to 12 kDa.<sup>36</sup> In its structure, this protein contains gamma-carboxy-glutamine acid and one disulphid connection.<sup>37</sup>

Osteocalcine is one of the most present proteins in bone matrix. This protein is located in extra cellular dentin matrix.<sup>38</sup> Osteocalcine is synthesized and secreted by odontoblast in vivo<sup>39,40</sup> and in vitro conditions.<sup>41,42</sup>

Functional importance of osteocalcine is in accordance with the characteristics of all the Gla proteins. Osteocalcine binds tightly hydroxy apatite and inhibits the formation of hydroxy apatite in vitro.<sup>20</sup> These data point to the possible role of osteocalcine as a negative regulator of bone tissue and dentin synthesis.<sup>43</sup> The function of this protein is still unclear. Being synthesized in high concentrations in non-skeleton tissues, MPG is a mineralization inhibitor.<sup>44,45</sup>

These proteins have anion character and are capable of binding calcium ions and thus it can be assumed that this protein group also has a very important role in mineralization.<sup>46</sup>

The research results done in experimental part of this work undoubtedly point to the presence of proteoglycan in dentin. Achieved results of proteoglycan molecular mass move about 75 kDa (Figure 3), which matches literary data.<sup>47</sup> Two most important small proteoglycans located in dentin are decorin and biglycan.<sup>48</sup> Literary data point to the fact that proteoglycans make secretion in predentin and in a way prevent the process of its mineralization.<sup>20</sup> Research shows that 25-50% of nascent proteoglycans is lost in the process of dentin synthesis.<sup>49</sup> Steinfort and associates<sup>50</sup> have proven rapid incorporation of proteoglycans in dentin while, at the same time, the quantity of proteins in predentin becomes less during dentinogenesis. The study by Hoshia and associates<sup>51</sup> shows that immediately before the mineralization, collagen fibres combine and become bigger in their diameter. By using detection through immunolocalization it has been found that together with collagen fibre fusion decorin concentration becomes less. The data of this study show that one of the important moments in the process of dentinogenesis is lowering the level of decorin proteoglycan with the aim of improving collagen fusion and mineralization induction.

Sposobnost nekih proteoglikana da se udružuju sa kolagenom sugerise njihov mugući doprinos u maturaciji kolagenih fibrila.<sup>5</sup> Proteoglikani kao što je hondroitin sulfat mogu inhibirati proces mineralizacije. Drugi proteoglikani mogu naspecifično vezati jone kalcijuma i indukovati formiranje hidroksiapatita in vitro. U organskom matriksu dentina podaci iz literature ukazuju da je identifikovano nekoliko glikozoaminoglikana – hondroitin-4-sulfat, hondroitin-6-sulfat, dermatin sulfat, heparin sulfat, keratin sulfat. Distribucija ovih proteoglikana u predentinu i dentinu je takođe bila analizirana. Otkriveno je da se u predentinu mogu locirati homogene, relativno velike i stabilne količine hondroitin-4-sulfata, dermatin sulfata i keratin sulfata. Koncentracija hondroitin-6-sulfata u predentinu je veoma mala. Distribucija proteoglikana u dentinu je heterogena. Jedino se hondroitin-4-sulfat može otkriti u mineralizovanom dentinu.<sup>2</sup>

Poznato je da postoji interakcija između proteoglikana i kolagena zbog čega se smatra da je jedna od mogućih uloga proteoglikana u dentinogenези да utiču na, ili čak kontrolišu organizaciju kolagene mreže formirane u predentinu.<sup>49</sup>

Kao i fosfoproteini, i proteoglikani imaju jaku izrazitu sposobnost vezivanja za jone kalcijuma. Sa tim u vezi dokazana je njihova induktivna uloga u sintezi hidroksiapatita in vitro pri fiziološkoj pH vrednosti i jonskoj koncentraciji (pH 7.0).<sup>50</sup>

## Zaključak

Rezultati dobijeni u ovom istraživanju potvrđuju da osnovnu nespecifičnu grupu nekolagenih proteina koji su produkt sekretorne aktivnosti odontoblasta čine kiseli fosfoproteini. Nađeni fosfoproteini imaju molekularnu masu od 35 do 158 kDa. Njihov veliki afinitet prema jonima kalcijuma implicira ulogu fosfoproteina u transportu jona ka mineralizacionom frontu.

Elektroforetička analiza frakcija dentina potvrdila je i prisustvo Gla proteina malih molekularnih masa i proteoglikana mase oko 75 kDa u dentinu.

Ability of some proteoglycans to combine with collagen suggests their possible contribution in maturation of collagen fibrilla.<sup>5</sup> Proteoglycans such as chondroitin sulphate can inhibit the mineralization process. Other proteoglycans can non-specifically bind calcium ions and induce hydroxy apatite formation in vitro. In organic dentin matrix, literary data points out that several glycosaminoglycans are identified – chondroitin-4-sulphate, chondroitin-6-sulphate, dermatane sulphate, heparin sulphate, keratan sulphate. The distribution of these proteoglycans in predentin and dentin has also been analyzed. It has been discovered that homogeneous, relatively big and stable quantities of chondroitin-4-sulphate, dermatane sulphate and keratan sulphate can be located in predentin. The concentration of chondroitin-6-sulphate in predentin is very small. The distribution of proteoglycan in dentin is heterogeneous. Only chondroitin-4-sulphate can be discovered in mineralized dentin.<sup>2</sup>

It is known that there is an interaction between proteo-glycon and collagen due to which it is considered that one of the possible roles of proteo-glycon in dentinogenesis is to influence or even control the organization of collagen network formed in predentin.<sup>49</sup>

As phosphoproteins, proteoglycans also have remarkable ability to bind with calcium ions. With that in view, their inductive role has been proven in the hydroxy apatite synthesis in vitro under physical pH value and ion concentration (pH7.0).<sup>50</sup>

## Conclusion

The results obtained in this research confirm that basic non-specific group of non-collagen proteins which are products of odontoblast secretory activity are in fact acid phosphoproteins. Found phosphoproteins have molecular mass from 35 to 158 kDa. Their big affinity towards calcium ions implies the role of phosphoproteins in ion transport towards mineralized front.

Electrophoretic analysis of dentin fractions confirms also the presence of Gla proteins of small molecular masses. The research has confirmed the presence of proteoglycan mass about 75 kDa in dentin.

Funkcionalni značaj Gla ptoteina se ogleda u tome što oni čvrsto vezuju hidroksiapatit i inhibitori su formiranja hidroksiapatita in vitro. Ovi podaci upućuju na moguću ulogu Gla proteina kao negativnog regulatora sinteze dentina.

Proteoglikani su nespecifični nekolageni proteini dentina. Njihova uloga u dentinogenezi je važna i nezamenljiva. Oni se sekretuju u predentin i na izvestan način sprečavaju proces njegove mineralizacije.

Analizirajući prethodno iznete podatke može se zaključiti da je međusobna interakcija svih pomenutih činilaca neophodan preduslov pravilnog procesa dentinogeneze.

Functional importance of Gla proteins is seen in the fact that they bind tightly hydroxylapatite and that they inhibit the formation of hydroxylapatite in vitro. These data point to the possible role of Gla protein as a negative regulator of dentin synthesis.

Proteoglycans are non-specific, non-collagen dentin proteins. Their role in dentin-gensis is very important and irreplaceable. They make secretion in predentin and in a way prevent its mineralization process.

By analyzing previously outlined data it can be concluded that mutual interaction of all the mentioned factors is a necessary prerequisite for dentinogenesis process.

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