Sorptive Isolation of Lysozyme from Hen Egg White

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Abstract. **Isolation of proteins from available biological resources is the matter of interest both for scientific and practical approach. To substitute conventional isolation methods by more selective sorption technique, appropriate sorbent has been used which was developed on the base of acrylic monomers and contained methacrylic and acrylic acid units. Sorption of lysozyme from model binary solution took place with high selectivity under experimental conditions, the results of which were used for lysozyme isolation from diluted hen egg white. Protein(s) sorption onto carboxylic ion-exchanger at optimal condition in static regime and desorption in dynamic downstream regime allowed isolating lysozyme containing fraction with lytic activity against *Micrococcus lysodeikticus* achieving 68.8 – 75% of the standard lysozyme activity.**

Keywords: **sorption, cation-exchanger, lysozyme, hen egg white, lytic activity**

I. Introduction

Isolation of valuable bio-components from available biological resources usually is a multistage process. Conventional methods for target protein isolation involve combination of precipitation, centrifugation and crystallization techniques. It is recognized that these techniques have poor selectivity and therefore have to be repeated several times to obtain reasonably pure protein [1]. The multistage process led to protein loss and large amount of waste solutions.

One of the most marketable bio-component is enzyme lysozyme. Lysozyme (muramidase, EC.3.2.1.17) has been found in a wide variety of biological objects. Lysozyme is a component of physiological fluids in mammalians, it is found in reptiles, insects, and plants. The content of lysozyme in hen egg white is large enough to isolate it in technological scale [2, 3]. Currently, lysozyme is used in pharmacology as a drug, in food technology as an antibacterial agent in some processes (cheese, beer and vine production) and for food preservation [4]. Antibacterial activity of lysozyme is expressed by hydrolysis of linkage between N-acetylmuramic acid and N-acetylglucosamine in mucopeptide wall structure of microbial cells.

In connection with a broad spectrum of lysozyme utilization, it can be exploited as enzyme of different purity. The degree of lysozyme purity is expressed, together with other methods, by its lytic activity against *Micrococcus lysodeiktikus*.

Recently, series of scientific works were devoted to lysozyme isolation from biological sources and to purification using chromatography (dye-ligand, hydrophobic, metal-ligand, ion-exchange) [5, 6, 7, 8] or membrane technology [9, 10].

Most of sorbents used for protein isolation are based upon permeable polysaccharide matrix. Such cellulose, dextran and agarose sorbents have usually low sorption capacity, large swelling, and low mechanical stability.

Chemical modification of natural material can diminish these disadvantages and allows using resulting material for isolation of lysozyme from native sources [11].

Polymeric materials are the most preferable sorbents for recovery and purification of biologically active compounds. There are high sorption capacities of these materials for biologically active compounds due to the polyfunctional nature of the interaction with target compounds [12]. The problem is to develop a functional polymeric material having appropriate functional groups and permeable matrix for large molecules. The polymeric beads must have spherical surface form without a dense layer on it, which is a hindrance for protein molecule diffusion into bead inside.

Earlier developed carboxylic cation-exchanger K-120 has an aliphatic matrix containing methacrylic and acrylic acid units. Spherical polymeric beads are permeable for protein molecules. The defined level of functional group ionization provides high binding capacity and reversibility. Enhanced content of cross-linking agent ensures appropriate osmotic stability of network [13].

The previous study of lysozyme sorption/desorption regularities onto synthesized ter-polymeric carboxylic cation-exchanger allowed elucidating optimal conditions for lysozyme isolation without the loss of its lytic activity [14]. In present work, the lysozyme isolation from hen egg white (HEW) has been studied using a combined technique: the sorption has taken place in batch contactor with agitation, but for desorption process a fixed-bed column has been used [15].

II. Materials and Methods

Carboxylic cation-exchanger K-120 has been synthesized as reported elsewhere [13]. Working fractions of polymeric dispersion were 0.315 – 0.5mm and 0.2 – 0.315 mm (for a combined process).

Lysozyme from hen egg white (Fluka), (M ~ 14600, IP 10.4) was dissolved in buffer solution, the concentration being 1 mg/ml or 2 mg/ml. 100 mg (on air-dried weight) of swollen equilibrated cation-exchanger was located into flasks containing 10 ml of a solution and shaken during definite time.

Protein sorption from binary solutions containing lysozyme and bovine serum albumin (BSA) (SIGMA) was conducted using 1 ml of sorbent K-120 equilibrated with fitting buffer solution and 10 ml of a sorption solution.

Desorption solution contained 0.2 M of phosphate salt and 0.3 M of NaCl, pH being 9.0. Desorption of lysozyme was done after washing of isolated beads with fitting buffer solution. Filtered samples were located in flasks containing 10 ml of desorption solution and shaken during 0.5 h.

Hen egg white was manually separated from yolk and diluted with 0.05 M phosphate buffer solution (PBS) containing 0.1 M NaCl and having pH value 7.0. The concentration of whole HEW in PBS was 25 %. The mixture was homogenized by mixing at magnetic stirrer 5 min and passed through nylon filter. pH value of working medium was 7.5.

Proteins sorption from HEW medium was performed using preliminary equilibrated sorbent volume and was calculated to cation-exchanger volume.

Sorption kinetics was studied by individual samples contacting with corresponding medium; the protein concentration and lytic activity were measured after each 30 min.

Lysozyme isolation from HEW was done under static conditions. Then the sorption cation-exchanger was filtered through a nylon filter, washed with PBS (8V/Vsorb.) and transferred into column (ID 1.4 cm, H 7.0 cm).

Desorption took place under dynamic conditions using a downstream regime. Desorption solution was passed through the column at a flow rate 7.5-7.8 ml/cm^2 h.

Sorption capacity was calculated as follows:

SC = (CxV) / mx[(100-W)x10^{-2}], mg/g; where C – protein concentration in desorbate, mg/ml; V – volume of desorbate, ml; m – air-dried sorbent mass, g; W – moisture content in the sorbent, %.

Protein concentration in the solutions was determined by Benedict method.

Lytic activity of lysozyme containing solutions was evaluated using *Micrococcus lysodeikticus* lyophilized cells (SIGMA) dispersed in 0.1 M PBS, pH 7.0. Optical density decrease was fixed during 3 min at 450 nm using Spectrophotometer YENWEY 6300.

The mechanical strength of cation-exchanger was determined as a response to compression. Swollen sorbent was inserted into a metallic cylinder fitted with a non-hermetic movable piston and mounted for compressive test to INSTRON 4301. Relationship between external pressure p = F/A and percentage of ΔV/ V was acquired (F – load, A – cross-section of piston, V – initial volume of sorbent and ΔV is the change in volume).

Synthesized ion-exchanger morphology was studied using Tescan Mira//LMU Schottky type electron microscope (SEM).

III. Results

Synthesized cation-exchanger K-120 contains a large number of functional groups – 10.2 mekv/g and moderate swell in water (specific swelling – 3.2 ml/g). Previous experiments showed that cation-exchanger presents high sorption capacity towards lysozyme [14]. In addition to high sorption capacity, selectivity of sorption is an exceedingly significant factor. Selectivity of sorption is governed by many factors. The effective sorption of desired protein from mixture of proteins depends not only on characteristics of independent proteins, ion-exchanger functional group nature, amount and condition, but also on low molecular weight component nature and concentration, longevity of process and so on. Separation of proteins can also be performed during a desorption stage.

Using the results obtained in the previous study, lysozyme isolation from model solution is to be conducted at pH ~ 7 and ionic strength ~ 0.15 M. The operation at these conditions allows obtaining the highest sorption capacity.

To elucidate the sorption gait with time, sorption kinetics has been studied from model solutions (Fig. 1). Lysozyme sorption from model solution containing 2 mg lysozyme/ml approaches the equilibrium state at 3 h under experimental conditions, but from solution containing 1 mg/ml – at 2 h.

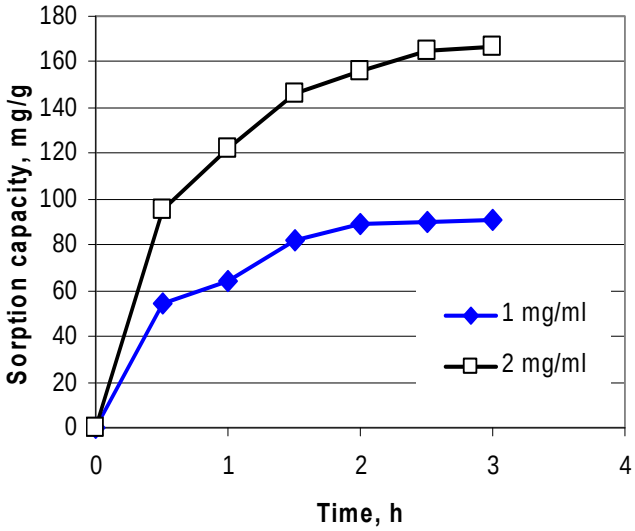

Fig. 1. Sorption of lysozyme from individual solutions

Sorption of proteins from binary solutions containing lysozyme and fivefold concentration of BSA at pH 7.0 and ionic strength 0.15 M was studied as dependence on time (Fig. 2). Protein concentration in desorbates reflected chosen relationship lysozyme: BSA. At 0.5 mg/ml : 2.5 mg/ml and 1.0 mg/ml : 5.0 mg/ml sorption process achieved equilibrium yet for 1 – 2 h and the protein concentration level allowed supposing that all lysozyme amount was captured by cation-exchanger.

Sorption from solution containing 2.0 mg lysozyme and 10.0 mg BSA in 1 ml approached equilibrium for 2 h.

The content of lysozyme in the obtained desorption solutions was evaluated via their lytic activity towards *Micrococcus lysodeikticus* cells (Fig.3). The results showed that lytic activity of desorbates obtained after sorption at 1.5 h from 0.5 mg/ml : 2.5 mg/ml and 1.0 mg/ml : 5.0 mg/ml solutions corresponded to lytic activity of the solution containing only lysozyme. Lytic activity of desorbate obtained after the sorption from 2.0 mg/ml : 10.0 mg/ml solution was smaller than activity of pure lysozyme solution. Together with lysozyme sorption, BSA sorption might be expected from this solution.

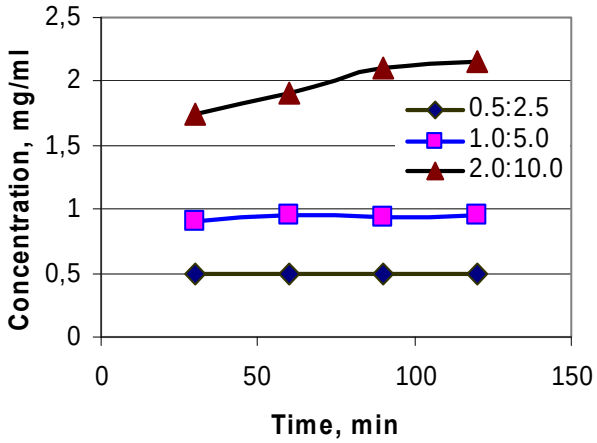

Fig. 2. Protein concentration in desorbates obtained after sorption from binary solutions; lysozyme : BSA concentration (mg/ml) is given in series

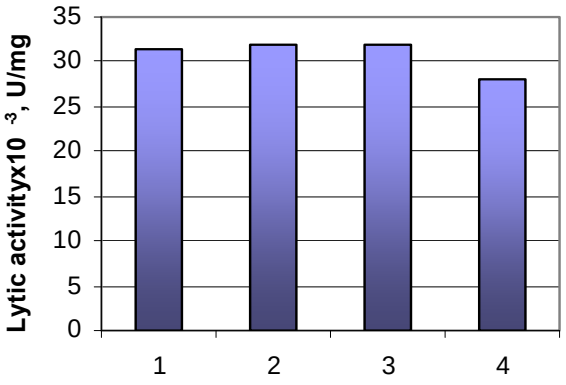

Fig. 3. Lytic activity of lysozyme solution (1) and desorbates obtained after sorption from lysozyme : BSA solutions (concentration: 2 - 0.5 mg/ml : 2.5 mg/ml; 3 - 1.0 mg/ml : 5.0 mg/ml; 4- 2.0 mg/ml : 10.0 mg/ml)

The data expressed in Fig. 2 and Fig. 3 testified high selectivity of lysozyme sorption under the chosen conditions. Similar conditions have been used for lysozyme sorption from HEW medium. Protein(s) sorption under static conditions approached equilibrium state at 1.5 h (Fig. 4). Lysozyme isolation from HEW was performed using a combined technique: i) sorption stage was done under static conditions in closed agitated contactor and ii) for desorption washed sorbent was transferred into a column, where protein(s) were eluted by downstream process.

Taking into account the practical aspect, experiments for lysozyme isolation from HEW have been conducted using the phase ratio between K-120 and HEW medium equal to 1 ml : 3 ml, 1ml : 5 ml and 1 ml : 8 ml. Sorption time was 1.5 h.

Then sorption sorbent was filtered, washed and transferred into a column. Elution of proteins was performed using a downstream regime. The content of proteins in eluted volumes corresponded to volumes taken for the sorption (Fig. 5).

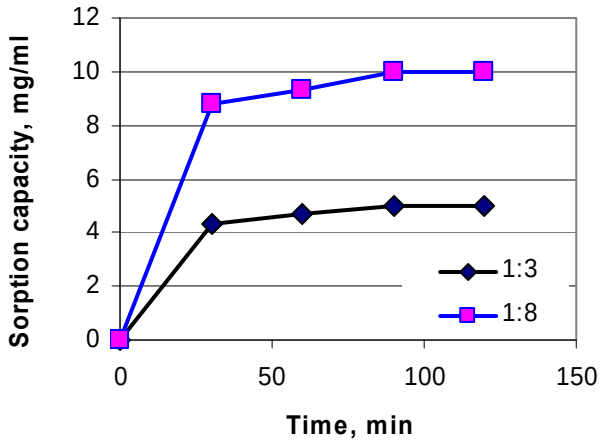

Fig. 4. Kinetics of protein sorption from HEW medium; K-120 volume : HEW medium volume is shown in series

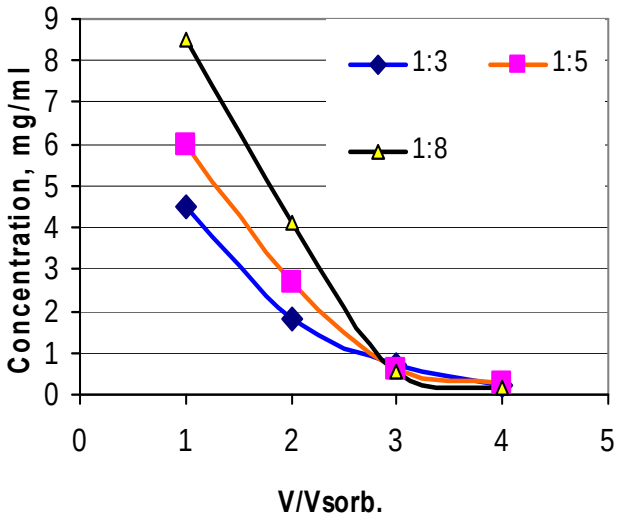

Fig. 5. Protein concentration in desorbates; in series – the ratio sorbent (ml) : sorption medium (ml)

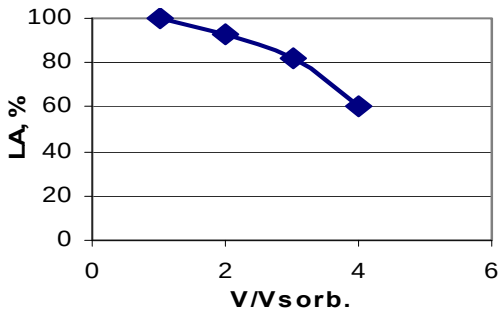

Fig. 6. Lytic activity (LA) of desorbates

The desorbed proteins were concentrated already in the first eluted volume after leaving the washing buffer. The content of lysozyme in eluted fractions was evaluated by their lytic activity against *Micrococcus lysodeiktikus* cells (Fig. 6). The most active was the first fraction.

Lytic activity of protein in the first fraction was $22x10^3$ – $24x10^3$ U/mg at all ratios tested, and taking into account that lytic activity of pure lysozyme was $32x10^3$ U/mg, the content of lysozyme in the first fraction was 68.8 – 75 %. Lytic activity of initial HEW medium was determined as $11x10^2$ U/mg, that is, the content of lysozyme in it was 3.4% that corresponded to the data provided in literature.

pH value of desorbates was approximately 7, but small change of it was noticed in desorbed volumes. The minimal pH was in the second fraction (6.5). The change of pH is connected with i) re-charging of functional groups, and ii) the change of lysozyme concentration.

The calculated output of isolated lysozyme naturally depended on the ratio of solute volume to sorbent volume. When the ratio between the sorbent volume and sorption medium volume was 1: 3, the output of lysozyme was ~ 70% in the first eluted volume and the ratio increase till 1: 8 diminished the output up to 54 – 59%.

From the practical aspect, the process reduplication is significant. As experiments showed, sorption capacity of cation-exchanger did not diminish after at least 5 cycles. Mechanical strength of cation-exchanger is also a consequential parameter for practical application of sorption material. To evaluate the mechanical resistance of the sorbent, the response to compression was examined. The amount of fine fraction after the test did not increase essentially after 5 cycles and composed only less than 5 %. Also this amount was not large when the sorbent with the proteins sorbet from HEW was tested (about 2.5 %). The diagram testified the absence of any noticeable destruction during compression (Fig. 7).

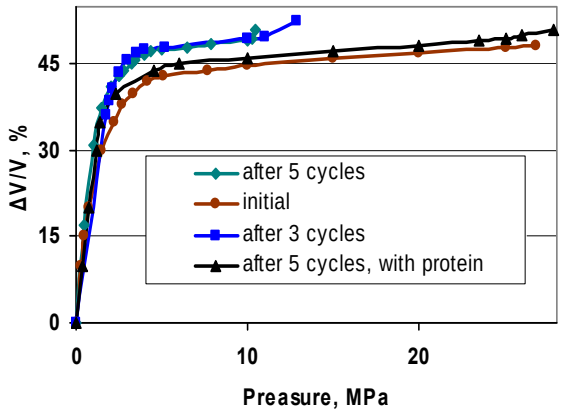

Fig. 7. Swollen sorbent volume change induced by external pressure

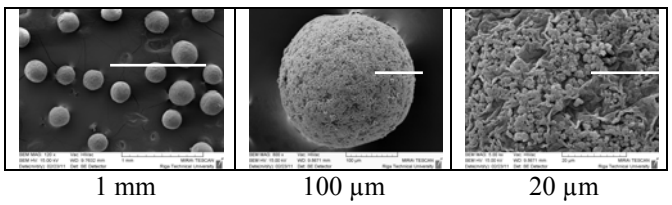

Fig. 8. Scanning electron microscopy of bead surface

After 5 cycles of sorption – desorption and mechanical test, the ion-exchanger beads preserved their spherical surface form and did not have any cracks.

Scanning electron microscopy testified macroporous structure of the beads formed by connecting micro-globules and permeable surface without dense cover on it (Fig. 8).

IV. Conclusions

1. Ter-polymeric carboxylic ion-exchanger containing methacrylic and acrylic acid units and triethyleneglycol dimethacrylate as a cross-linking agent possessed high selectivity in lysozyme sorption process from binary solution. Under experimental conditions, the sorption of lysozyme from the solutions containing 0.5 or 1.0 mg/ml lysozyme and 2.5 or 5.0 mg/ml BSA was practically quantitative at 30-90 min. The increase in lysozyme and BSA concentration till 2.0 mg/ml and 10.0 mg/ml, correspondingly, diminished the lysozyme isolation till 92%.
2. Lysozyme isolation from diluted hen egg white can be successfully performed using a combined technique: sorption under static conditions in batch with agitation and desorption under dynamic conditions in a column using a downstream regime.
3. Lytic activity of desorbates against *Micrococcus lysodeiktikus* cells was 68.8 – 75 % from the activity of pure lysozyme.
4. Mechanical strength of swollen carboxylic cation-exchanger was defined by tests in compression. More than 95% of material after repeated sorption-desorption cycles could resist 45 MPa of external pressure.

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Valentina Krilova, Visvaldis Vītiņš. Līzocīma sorbtīvā izdalīšana no vistu olu baltuma
Vērtīgu bio-komponentu izdalīšana no pieejama bioloģiskā izejmateriāla parasti notiek kā daudzpakāpju process, kurā izgulsnēšana, kristalizācija, gēl-filtrācija u.c. daudzkārt atkārtojas lai iegūt pietiekamas tīrības mērķa produktu.
Līzocīms ir antibakteriālais enzīms, kuru lieto dažādos tehnoloģiskajos procesos. Tas ir atrasts veselā virknē bioloģisko objektu: dzīvnieku un cilvēka fizioloģiskajos šķidrumos, olu baltumā, dažos augos u.t.t. Līzocīma saturs olu baltumā pietiekoši liels (~ 3.4 %), un šo resursu izmanto fermenta tehnoloģiskai izdalīšanai.
Dotajā darbā izpētīta līzocīma izdalīšana no olu baltuma pielietojot sintezētu karboksilkatjonītu – metakrilskābes, akrilskābes un trietilēnglikoldimetakrilāta ter- polimēru. Karboksilkatjonīta porainās sfēriskās granulas ir caurlaidīgas proteīna molekulām. Līzocīma sorbcija pētīta statiskajos apstākļos 0.05 M fosfāta buferšķīdumā ar pH 7, kurš satur 0.1 M NaCl. Līzocīma izdalīšana no modeļa bināra šķīduma notika ar augstu selektivitāti.
Līzocīma izdalīšana no olu baltuma veikta kombinētā režīmā: sorbcija īstenojas statiskajā režīmā ar maisīšanu, bet desorbcija – dināmiskajā režīmā kolonā ar nekustīgu slāni. Desorbcija notika 0.2 M fosfāta šķīdumā ar 0.3 M NaCl (pH 9). Desorbātu pH lielums svārstās no 6.5 līdz 6.9. Proteīns maksimāli koncentrējas pirmajā izlaista desorbāta tilpumā.
Šķīdumu enzimātiskā aktivitāte noteikta pēc *Micrococcus lysodeiktikus* šūnu lītiskās reakcijas. Līstiskā aktivitāte bija maksimāla pirmajā desorbāta tilpumā un tā lielums sastādīja 68.8-75 % no standarta līzocīma aktivitātes.
Hidratētu paraugu (pH 7) mehāniskās stiprības novērtēšanai pēc piecu ciklu īstenošanas tika veikti kompresijas testi uz INSTRON-1011. Pielietojot slodzi līdz 45 MPa materiāla smalka frakcija sastādīja mazāk par 5 %.

Валентина Крылова, Висвалдис Витыньш. Сорбционное выделение лизоцима из яичного белка.
Выделение ценных био-компонентов из доступного биологического сырья обычно является многоступенчатым процессом, в котором осаждение, кристаллизация, гель-фильтрация и т.д. многократно повторяются для получения целевого продукта достаточной чистоты.
Лизоцим является антибактериальным ферментом, используемым в различных технологических процессах. Он обнаружен в целом ряде биологических объектов: в физиологических жидкостях млекопитающих и человека, в яичном белке, в некоторых растениях и т.д. Содержание лизоцима в яичном белке достаточно высоко (3.4 %), и этот ресурс используется для технологического выделения фермента.
В данной работе изучен процесс выделения лизоцима из яичного белка с использованием синтезированного карбоксильного катионита - тер-полимера метакриловой, акриловой кислот и триэтиленгликольдиметакрилата. Пористые сферические гранулы карбоксильного катионита являются проницаемыми для молекул белка. Сорбция лизоцима изучалась в статических условиях в 0.05 М фосфатном буферном растворе, содержащим 0.1 М хлорида натрия и имеющим pH 7. Сорбция лизоцима из модельного бинарного раствора проходила с высокой степенью селективности. Выделение лизоцима из яичного белка проводилось в смешанном режиме: сорбция - в статическом режиме с перемешиванием, десорбция - в динамическом режиме в колонке с неподвижным слоем. Десорбция осуществлялась 0.2 М фосфатным раствором, содержащим 0.3 М хлорида натрия (pH 9). При этом pH десорбатов менялось от 6.5 до 6.9. Содержание белка было максимальным в первом пропущенном объёме.
Ензиматическая активность растворов определена по лизису клеток *Micrococcus lysodektricus*. Литическая активность была наивысшей в первом объёме десорбата и составляла величину 68.8-75 % от величины стандартного лизоцима.
Для оценки механической прочности гидратированных образцов сорбента при pH 7 после 5-кратного их применения проведены тесты на компрессию с использованием INSTRON -1011. При нагрузке 45 МПа отсев материала составлял < 5 %.