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Research Article

A Simple, Cheap and Fast Method for Calculation of Peptides to be used as Ligands in Affinity Chromatography or in Other Applications in Biotech and Pharma Industry

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ABSTRACT

"The Proteome Code" concept introduced by J. Biro' demonstrated that it has a very high potential to become a fast, inexpensive, highly productive technology for affinity peptides producing, alternative to the methods of generating antibodies *in vivo* including the different many screening methods such as combinatorial chemistry, mRNA display, phage display, and computer-based virtual screening technology.

Developing J. Biro' method we created of a simplified algorithm for calculating the affinity peptide to the target protein and to practical verification of the possibility of applying the concept of "The Proteome Code".

This development was built on an example of chromatographic gel synthesis for coagulation factors VIII and X purification based on affinity peptides. It was also important to figure out how simple, fast, and costly this development could be.

Keywords: Affinity Peptides; FVIII; FX; Chromatography Ligands

ABBREVIATIONS

TNBP: three-*n*-butyl-phosphate; *h*FVIII: human Factor VIII; *h*FIX(a): (activated) human Factor IX; *h*FX(a): (activated) human Factor X; *h*VWF: human von Willebrand Factor; PEG: polyethylene glycol: TFA: trifluoroacetic acid; EDT: ethanedithiol; DBC: dynamic binding capacity; $aa_{(s)}$: amino acid(s);

INTRODUCTION

The beginning of affinity peptides use on a production scale can be considered by Smith [1] publication in 1985 describing the phage display method. It allowed to create large libraries of nucleotides encoding polypeptides and to quick analyze the suitability of the latter for affinity ligand applications. Until today this method has been improved [2-4] and transformed into a technology of new peptide ligands production for proteins chromatographic purification [5-9]. The final stage of peptide ligands implementation in industrial affinity chromatography was the validation of peptide gel performed for cGMP FVIII industrial-scale chromatographic purification [10]. In the course of studies, the peptide ligand stable coupling to the chromatographic matrix, the range of effective dynamic capacity, the stability of the protein binding (not less than 75 chromatographic cycles) and the purification efficiency of the target protein were shown. But the most important were: 1) strong stability peptide ligand under extreme purification conditions, for example, guanidine chloride at low pH, 2) high tolerance level to treatment with virus-inactivating reagents (TNBP, Triton X-100) at reasonable concentration, 3) the leaching of the peptide ligand from the chromatographic matrix was very small – 3×10^6 times less than the volume causing any reaction of animal cell, 4) free leaching of the peptide was effectively preserve by the adsorbent storage at 2-8 °C, and the released peptide was effectively removed by column washing with a buffer containing low concentration of ethylene glycol or by subsequent separation of the target protein and peptide on the column with any other type of chromatographic adsorbent. Thus, the "right to life" of peptide affinity gel in biotechnology industry was confirmed [10].

To obtain the top performing affinity peptides rapidly, many screening methods have been developed such as combinatorial chemistry [11-12], mRNA display [13-14], phage display [15], and computer-based virtual screening technology [16]. Most of them are highly dependent on high-throughput experimental screening, that results in heavy workload [11-16]. Instead the structure-based molecular docking method has recently gained momentum and great support due to possibility to solve the problems of operation simplicity, reducing the intensity of peptide screening, and shortening the development cycle [17-18]. All above also makes the peptide developing method much cheaper, what is one of the indicators of its manufacturability [19-20].

To the structure-based molecular docking method we are including, with some remarks and caution, the *Biro*' publications, who began the method developing from considering of co-located amino acids a physicochemical interaction in the process of protein-protein affinity sites recognition. As shown, this interaction of proteins was embedded in complementary sense-antisense nucleotide sequences encoding amino acids [21-23]. So, studies of the possible role of antisense peptides in molecular interaction have been carried out [24-26] and the formulation of the concept "The Proteome Code", which allows molecular recognition of the protein code, was completed by 2011 [27-29].

"The Proteome Code" concept demonstrated that (*further the author's text* [28]) "…1) codons developed in association with encoded amino acids; there is a stereochemical connection between several (if not all) amino acids and their codons; 2) wobble bases are not randomly chosen in synonymous codons, but each has well defined role in determining the structure of nucleic acids and their folding energies, 3) co-locating amino acid are preferentially encoded by partially complementary codons, where the 1st and 3rd codon residues are complementary to each other in reverse orientation, but the 2nd codon residues may but not necessarily do complement one another, 4) structural information contained within nucleic acids is transferred to proteins during translation, this transfer requires direct contact between "dedicated" amino acids and their codons – this process is called nucleic acid assisted protein folding or the concept of mRNA chaperons, 5) there is a tRNA cycle that allows direct codon amino acid contact to be possible".

The practical results of "The Proteome Code" concept were the creation of tables of sense-antisense amino acid pairs, which when co-located mainly interact with each other [25,30-31], and the development of the method for obtaining oligopeptides high affinity to target proteins [32].

The *Biro*' method had a very high potential to become a fast, inexpensive, highly productive technology for affinity peptides producing, alternative to the method of generating antibodies *in vivo* [32]. Unfortunately, after 2012 there are only a few publications with its partial using [33-34].

Thus, the present study was dedicated to creation of a simplified algorithm for calculating the affinity peptide to the target protein and to practical verification of the possibility of applying the concept of "The Proteome Code". This development was built on an example of chromatographic gel synthesis for coagulation factors VIII and X

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purific ation based on affi nity peptides. It was also important to figure out how simple, fast, and costly this development could be.

MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich (Green Chemistry LLC, distributor in Mongolia) unless otherwise indicated. Reagents and equipment from other manufacturers are indicated below. Human (donor's) plasma, obtained by plasmapheresis, was bought from Ulaanbaatar blood collection center (Mongolia).

To identify the human Factors VIII (*h*FVIII), X (*h*FX), and IX (*h*FIX) and to determine their concentrations Abcam (ELISE kits ab272771, ab188393, ab108832, respectively), and Diapharma Group, Inc., USA chromogenic kits (K824086, 900020, KDPGFX, respectively) were used. Th e measurement was carried out on Biotrak II Visible Plate Reader or Ultrospec 3000pro UV/VIS (GE Healthcare AB, Sweden).

The total protein in the collected samples was determined by Bradford method [35] with Stoscheck modification[36].

FVIII, FIX and FX were purifi ed from human plasma: FVIII – by our own method [37], FIX and FX – by the method Arrigi et al. [38]. For additional purifi cation of FX from traces of prothrombin we used a simple modifi cation – the last stage of separation was carried out by metal chelate chromatography according to Lloyd and Feldman [39]. Th e purity of obtained coagulation factors was controlled by 1D/2D electrophoresis at native and denatured condition [38-39]. Th e minimum of factors purity was 98,7-99,2%. Chromatographic process was performed on the systems ÄKTAexplorer and ÄKTAbasic 100 with integrated soft w are UNICORN 5.01; electrophoretic 1D analysis – on the SE 660 Dual Cooled Vertical Unit, 2D analysis – on the Ettan IPGphor II Isoelectric Focusing system and Multiphor II Electrophoresis system; the automatic visualization station – Processor Plus, and electrophoregram analysis system – Typhoon Trio Variable Mode Imager with Image Master 2D Platinum 6.0 DIGE software. All the hardware/software, equipment, chromatographic columns and gels, electrophoresis reagents, standards and kits purchased from GE Healthcare AB, Sweden.

Peptide array library

Peptide array library was created or rather «theoretically calculated» according to 7 developed principles described in the chapter "Results…" using nucleotide sequence of the human gene of FX, and amino acid (aa) sequences of the *h*FVIII and *h*FX from *Protein Knowledgebase - UPKB (UniProtKB/Swiss-Prot)*. For the library creation we have used published research/developments of the FVIII and FX interactions or establishment of competitive high affinity peptides-antagonists of FVIII/FX (K_d=1-100 nmol).

Array peptides synthesis

The peptide's array library, containing calculated affinity members (~50-100 nmol per spot), was synthesized (each peptide sequences on 5 spots) and simultaneously anchored through the C-termini on PEG-derivatized membranes Amino-PEG500–UC540 Sheet, using Fmoc chemistry and MultiPep SPOT synthesizer (membranes and equipment from Intavis AG, Germany). All peptides were chemically synthesized with short aa spacer GPGPG. For controls 5 spots in the random membrane places were occupied by spacer alone. Another 5 spots of the negative control in the random membrane places were erected by each mutant aa sequences (aa_s): A/R or A/K or A/H.

SPOT peptide array experiments

Briefly, the peptide-membrane after synthesis was twice washed with 20 mM Tris/Cl buffer, pH 7.4, including lower than 0.001 mM Na^+ (control by MGA-1000 Graphite Furnace Atomic Absorption Spectrometer, Beijing Lumex Analytical Equipment Co. Ltd., China), 150 mM choline chloride, 5 mM $MgCl_2$, 10% sucrose and 0.015% *n*-DDM, then overlaid for 5 min at 20 °C with fresh chromatographic purified FVIII or FX, diluted with washing buffer to 0,01 mM solution. In other experiments peptide-membrane was incubated with 0.01 mM FIX solution or with human plasma diluted 5 times by citrate buffer. After 3 washing of unbonded FIX, FX, peptides or plasma (2 min each) reactive spots were visualized with Typhoon Trio, λ_{Ex}=295nm, λ_{Em}=340 nm.

Then peptide-membrane was sequentially incubated for 3 min at 20 °C with citric acid-sodium citrate buffer at different pH starting at pH 5.00 till pH 3.00 with 0.05 unit increments. The steps gradient pH was made with Orion Star 9102 pH Automated Titrator (Thermo Fisher Scientific, China). After each washing the fluorescence detection were carried out. The affinity was determined by the binding strength of the investigated peptide or protein with peptide synthetized on membrane according to the buffer pH value at which 50% of investigated peptide was desorbed ($pH_{50\%}$).

The apparent Kd

The apparent K_d of each protein-protein, protein-peptide and peptide-peptide interaction was determined by standard procedure described by Baja et al. [40] for intact FVIII and FIX interactions with our modification for variable binding substances concentrations under constant FIX concentration.

Fmoc solid phase peptide synthesis

~0.5 mmol/~350 mg of each 15-mer peptides (10-mer affinity peptide plus 5-mer spacer) which were chosen as affinity ligands were synthesized using Fmoc chemistry and MultiPep CF station with single column module (Intavis AG, Germany). The assembled peptide was subsequently de-protected with reagent R (15 ml of 90% TFA, 5% thioanisol, 3% EDT, and 2% anisole), precipitated with cold diethyl ether and finally purified by reversed-phase flash chromatography (Biotage Selekt Flash Purification System, Sfär C18 D column; Biotage Trading Co. Ltd, China). Peptides synthesis was confirmed by MALDI-TOF mass spectrometry (contract order Biotechnology institute, Mongolia).

Peptide-affinity chromatographic gel synthesis

250 mg of peptide were dissolved in 25 mL sodium carbonate-bicarbonate buffer, pH 9.4, and the solution was pumped with flow 1 mL×min^{-1} through tandem of 5 mL columns BabyBio ACT (Bio-Works, Sweden) during 48 hours at 4 °C. The peptide density on the WorkBeads gel was determined by quantifying the difference between total peptide quantity in start solution and unbound peptide by fluorescence of tryptophan in the spacer measured by Typhoon Trio. All other manipulations, including blocking unreacted active groups, were done according to instruction of manufacturer [41]. Finally, the column tandem was rinsed with 250 ml of deionized water to remove the blocking agent and, before using, – with working buffer for equilibration or, before storage, – with 20% ethanol.

Determination of gel dynamic binding capacity

0.5 mL of peptide-affinity gel packed into 0.8 cm diameter, 1.0

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cm height column was equilibrated with 10 Vc citric acid-Na_2HPO_4 buffer, pH 7.4, and 1 Vc of 10 mg×mL^{-1} FVIII/FX in equilibration buffer was applied into column with flow 0.002-0.25 mL×min^{-1} (2-25 min residence time). Dynamic binding capacity (DBC) was determined at 10% breakthrough [42]. The column was washed with equilibration buffer, and the elution was performed with citric acid-Na_2HPO_4 buffer, pH 2.6. The washing and elution flow rate was 0.25 mL×min^{-1}.

FVIII and FX peptide-affinity purification from human plasma

As a sample for affinity purification of FVIII FVIII-reach high molecular protein fraction obtained from human plasma was used [37]; as FX sample served enriched fraction of clotting factors obtained after AEX Sepharose FF (GE Healthcare AB, Sweden) [43] captured from low molecular protein fraction [37]. Eluate was subjected to membrane filtration to achieve protein concentration 5 mg×mL^{-1}. Both factors (10 ml) were loaded onto BabyBio peptide-affinity columns equilibrated with 10 Vc of 20 mM citric acid-Na_2HPO_4 buffer, pH 7.4. The equilibration flow rate was 0,5 Vc×min^{-1}, the application – 0,1 Vc×min^{-1}. First washing: 10 Vc of equilibration buffer (1 Vc×min^{-1}); second – 5 Vc of equilibration buffer including 200 mM NaCl, pH 7.4, 0.5 Vc×min^{-1}. Elution was performed by 2 Vc of 20 mM citric acid-Na_2HPO_4 buffer, pH 2.6, 0.04 Vc×min^{-1}. Eluted fraction was collected for purity and yield of target proteins and impurities were determined by ELISA, electrophoresis, and HPLC analysis. Column regeneration and sanitization were performed with 2 Vc of 0.1 M glycine-HCl, pH 2.5, and 2 Vc of 0.05 M NaOH with flow rate 0.5 Vc×min^{-1}.

Peptide-affinity chromatographic gel life-time determination

The process described above was applied to ACTAexplorer for automatic 250 cycles. The recording of cycles 1, 25, 50, 100, 150, 200, and 250 were compared, and the yield and purity of the obtained FVIII/FX were measured.

FVIII/FX identification and purity determination after chromatography on peptide-affinity gel

Both factors concentration in collected chromatographic fraction were determined by Elisa and/or chromogenic kits accordingly to total protein quantity [35,36]. To do so, fractionated factors were additionally identified on the SDS-PAGE plate (4-20%) developed under nonreducing conditions and stained by fluorescein conjugated [44] Abcam rabbit polyclonal anti-*h*FVIII (ab236284), anti-*h*VWF (ab9378) and anti-*h*FX (ab 79929) antibodies.

Statistical analysis

The statistical processing of results was carried out by standard methods [45]. A value of $p<0.05$ was considered statistically significant. Data was presented as a mean ± Standard Errors (SEM) of at least 5 independent experiments unless otherwise indicated.

RESULTS AND DISCUSSION

Algorithm of peptide affinity calculation

At the beginning of the affinity peptide development the original aa sequence was increased up to 20 residues to rise a degree of the future interaction with the affinity peptide or lowered up to 5 residues to reduce time and resources for compiling and synthesizing a library of affinity peptides. During the selection of the sequence, we tried to include in its composition as much as possible α-, β- and γ-skeletal aa_s present in the overall sequence of the protein, and formed a library of affinity peptides in accordance with the following developed principles:

1. **Determine α-skeletal** aa_s (having only one antisense aa in the both directions 3'→5' and 5'→3') in the original sequence of the target protein, which may be Q, M, or W;
2. **Determine β-skeletal** aa_s (having one antisense aa in the direction 3'→5' and two antisense aa_s in the direction 5'→3', one of them shall be same as 3'→5', excluding the case of D) in the original sequence of the target protein, which may be C, E, H, K, or D;
3. **Determine γ-skeletal** aa_s (having one antisense aa in the direction 3'→5' and three or four antisense aa_s in the direction 5'→3', one of them shall be same as 3'→5') are determine in the original sequence of the target protein, which may be P, I, A, or G;
4. **Determine basic** aa_s (having two antisense aa_s in the both direction 3'→5' and 5'→3', one same one different, excluding the case of N) in the original sequence of the target protein, which may be F, Y, or N;
5. **Determine invariant** aa_s (having two or three, the case of L, antisense aa_s in the direction 3'→5', and three-four – in the direction 5'→3', one of them shall be same as 3'→5') in the original sequence of the target protein, which may be R, V, T, S, or L;
6. First make the 3'→5' peptide antisense sequence, starting with well-defined α-skeletal, then β- and γ-skeletal, basic and finally invariant aa_s; in the same way make 5'→3' sequence;
7. The next step is an assembling of 3'→5' and 5'→3' peptide sequences by selecting pairs of co-located aa_s by physicochemical properties, sequentially changing the β-, γ-skeletal, basic and invariant aa_s. Thus, all possible variants of peptide sequences will make the complete library;
8. Taking into account additional data, such as a) nucleotide sequence of the protein of interest gene; b) confirmed aa sequence of the target protein; c) data about research/development of the target protein highly specific (K_d=1-100 nmol) interactions with other proteins and/or peptides will significantly reduce the time and cost of material resources for the library completing.

An example of the affinity peptide's library development

As exemplary samples for the development of an affinity ligand, we have used coagulation FVIII and FX. FVIII is vital for the treatment of hemophilia. FX is not a critical protein in the hemostasis system and is not usually used as a drug, but its activated form is indispensable in the biotechnological process as 6×His-protease [46]. FVIII functions as a cofactor in the Xase complex responsible for anionic phospholipid surface-dependent conversion of FX to activated FX (FXa) by FIXa [47]. Moreover, the activation of FX occurs on the surface of FVIIIa, i.e. on the affinity peptide site(s) for FX [48].

In support of point 8 of the "Algorithm…" scientific literature offered us more than 25 sources of data important for library development. For example, the manuscript Takeyama and co-workers

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[47] shows that the FVIII structure model indicates possible FX-binding site(s) on residues [400-429] at A2 domain. Here and below if the sequence is inserted in square brackets a numeration corresponds to the protein without signal peptide. According to precursor sequence numeration which we use these residues match to 419-448 aa that bound to FX with K_d=~63 nM [48]. One peptide corresponding to aa residues [400-409], ^{419}LAPDDRSYKS^{428}, competitively inhibited both the FVIII-FX and FVIIIa/FXa-dependent binding. Additionally, it was shown that single ^{427}K/A and double ^{427}K/A, ^{428}S/A peptide mutations had approximately fourfold higher K_d value than wild-type FVIII.

It's clear that these FVIII-derived peptides must have the corresponding antisense peptides on FX, so that two proteins interaction may occur. For subsequent calculations, it is important that the antisense aa sequence is present in the linear FX fragment at least partially.

Table 1 demonstrates the method of appropriate natural peptide identification on *h*FX. On the first stage, the determination of the possible codons sequence of cDNA (or mRNA – not shown) is performed, second stage – is a writing antisense codon sequence, and third stage – the determination of the predicted antisense aa – is performed according to the Biro' tables of sense-antisense codons and aa_s [25,30-31].

On the last stage, the comparison of the theoretically calculated antisense sequence of codons and aa with the available in the databases (UPKB) nucleotide sequence of the FX gene and the aa sequence of the same protein is leading us to determination of natural aa sequence (or its part) or site of interaction with FVIII-derived peptide ^{419}LAPDDRSYKS^{428}.

Discovered in the FX aa sequence original peptide ^{357}KTGIVSGFGR^{366} was highly homologous to the theoretically calculated peptides NRGLLSRMFR (direction 3'→5') and KSGIVSRILR (direction 5'→3'). Keeping in mind the identical (green fields, Table 1) and corresponding by physicochemical properties (blue, dark blue and yellow fields) aa_s of the calculated peptides, their homology with the natural peptide was reached 90%. Just one aa R (rose field) did not coincide with the natural one, ^{363}G, which is occupying this position at the original peptide.

All twenty aa_s were calculated according to shown method and the Figure 1 of the sense-antisense aa_s was completed. At this stage it is

Table 1: Steps of theoretical calculation of the target affinity sequence on the *h*FX to the peptide ^{419}L-S^{428} of the *h*FVIII.

aa position number		416	417	418	419	420	421	422	423	424	425	426	427	428
*h*FVIII aa sequence in literature		**P**	**L**	**V**	**L**	**A**	**P**	**D**	**D**	**R**	**S**	**Y**	**K**	**S**
aa codons		CCT	CTT	GTT	CTT	GCT	CCT	GAT	GAT	CGT	AGT	TAT	AAA	AGT
		CCC	CTC	GTC	CTC	GCC	CCC	GAC	GAC	CGC	AGC	TAC	AAG	AGC
		CCA	CTA	GTA	CTA	GCA	CCA			CGA	TCG			TCG
		CCG	CTG	GTG	CTG	GCG	CCG			CGG	TCA			TCA
			TTG		TTG					AGA	TCT			TCT
										AGG	TCC			TCC
antisense codon 3'→5'		GGA	GAA	CAA	GAA	CGA	GGA	CTA	CTA	GCA	ACT	ATA	TTT	ACT
		GGG	GAG	CAG	GAG	CGG	GGG	CTG	CTG	GCG	GCT	ATG	TTC	GCT
		GGC	GAT	CAT	GAT	CGT	GGC			GCT	CGA			CGA
		GGT	GAC	CTC	GAC	CGC	GGT			GCC	TGA			TGA
			AAC		AAC					TCT	AGA			AGA
										TCC	GGA			GGA
calculated antisense aa sequence 3'→5'		G	E	Q	E	R	G	L	L	A	R	I	F	R
			D	H	D					S	S	M		S
			N		N						stop			stop
antisense codon 5'→3'		AGG	AAG	AAC	AAG	AGC	AGG	ATC	ATC	ACG	ACT	ATA	TTT	ACT
		CGG	GAG	GAC	GAG	GGC	CGG	GTC	GTC	GCG	GCT	GTA	CTT	GCT
		GGG	TAG	TAC	TAG	TGC	GGG			TCG	CGA			CGA
		TGG	CAG	CAC	CAG	GAT	TGG			CCG	TGA			TGA
			CAA		CAA	CGC				TCT	AGA			AGA
										CCT	GGA			GGA
calculated antisense aa sequence 5'→3'		R	K	N	K	S	R	I	I	A	T	I	F	T
		G	E	D	E	G	G	V	V	P	A	V	L	A
		W	stop	Y	stop	C	W			S	R			R
			Q	H	Q	R				T	stop			stop
aa position number		354	355	356	357	358	359	360	361	362	363	364	365	366
*h*FX aa sequence from UPKB		**M**	**T**	**Q**	**K**	**T**	**G**	**I**	**V**	**S**	**G**	**F**	**G**	**R**
resulting aa sequence	3'→5'	**G**	**N**	**Q**	**N**	**R**	**G**	**L**	**L**	**S**	**R**	**M**	**F**	**R**
	5'→3'	**G**	**Q**	**H**	**K**	**S**	**G**	**I**	**V**	**S**	**R**	**I**	**L**	**R**

Note 1. The red letters – Stop codons.

Note 2. Green box – aa_s identical to aa_s of natural hFX-derived peptide, light blue box - aa_s closed by physicochemical properties, yellow box – closed, but weakly charged, dark blue box – closed, but strongly charged.

Note 3. The violet box – additional three aa_s to the proposed by Takeyama and co-workers [47], which had highly homology with aa_s of natural hFX-derived peptide.

Abbreviations: *h*FVIII: Human Factor VIII; *h*FX: Human Factor X; UPKB: Protein Knowledgebase UniProtKB/Swiss-Prot; aa: Amino Acid

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not necessary to calculate antisense aa_s through nucleotides sequence, but to wright down the antisense aa for both directions using Figure 1. For the simplicity of the peptide visualization we schematically showed the charge, hydropathy and "size", as MW derivative, of aa_s at the figure.

The searching for the affinity peptide to ^{419}L-S^{428} in the FX sequence revealed that at least three more aa_s, in both FVIII and FX, can contribute to the interaction of these proteins (violet field, Table 1). Moreover, the calculated antisense sequences were also identical or had very close properties to those in natural peptides. Our further research used the sense-antisense peptides extended to these three aa_s: ^{416}P-S^{428} from *h*FVIII and ^{354}M-R^{366} from *h*FX.

In the study of interaction of FX-derived peptide ^{354}M-R^{366} and two calculated peptides GNQNRGLLSRMFR (No.1)/GQHKSGIVSRILR (No.2) with the *h*FVIII and vice versa the ^{416}P-S^{428} with the *h*FX was established that all examined peptides had high affinity to their proteins (each K_d was not higher than 56 nM). The *h*FVIII-*h*FX interaction had high affinity too (K_d=28 nM). These results suggest that sites of factors interaction were found correctly. This conclusion is also confirmed by the unidirectional process of FVIII desorption from peptides No. 1 and 2, but not from peptide ^{354}M-R^{366} offered in paper [47-48]. If the $pH_{50\%}$ for peptides No. 1 and 2 was almost the same (~3.848 and ~3.852, respectively), in the last case $pH_{50\%}$ was 3.97 only. The affinity of the ^{419}L-S^{428} peptide to FX (Figure. 2) also left much to be desired ($pH_{50\%}$=3.91). In any case, it should be noted that the determined values of $pH_{50\%}$ are true to high-affinity peptide-peptide or peptide-protein interaction, where the $pH_{50\%}$ is usually in the range 3.0-4.0 as, for example, in the interaction of Protein A or G with IgG [49]. Importantly, that experiments with gradual decrease of the buffer pH allowed obtaining $pH_{50\%}$ values that correlated well with the calculated K_d and made it possible to assess the affinity level of the protein-ligand interaction.

Peptide array libraries creation

FX binding site located on the FVIII sequence ^{416}P-S^{428} helped to recognize the binding site for the FVIII on the FX. Both sets of proteins-derived peptides revealed good affinity, which, however, requires a significant improvement to allow peptides serve as affinity ligands for chromatographic purification of the FVIII and FX.

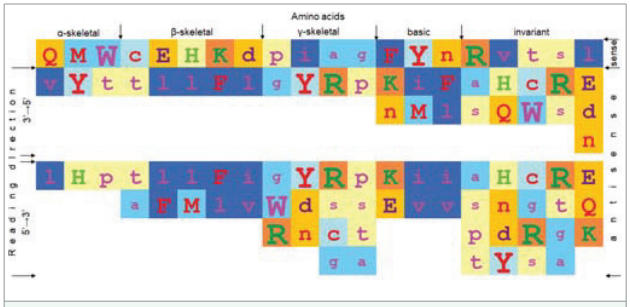

Figure 1: Sense-antisense amino acids for the both 3'→5' and 5'→3' directions of reading. All aa_s divided according MW (too small letters - MW≤105 Da, small letters – 115÷133 Da, big letters – 146÷165 Da, too big letters – 174÷204 Da), hydrophobicity (light blue box – light HPI=0.1÷0.2, blue box – medium 0.7÷1.5, dark blue box – high 2.2÷3.1), hydrophilicity (light yellow box – light HPI= –0.3÷–1.7, yellow box – medium –2.6÷–3.0, orange box – high –4.6÷–7.5), and charge under media pH=7.4 (light positive – light green letter, high positive – green letters, negative: light – light pink letters, medium – red letters, high – dark purple letters).

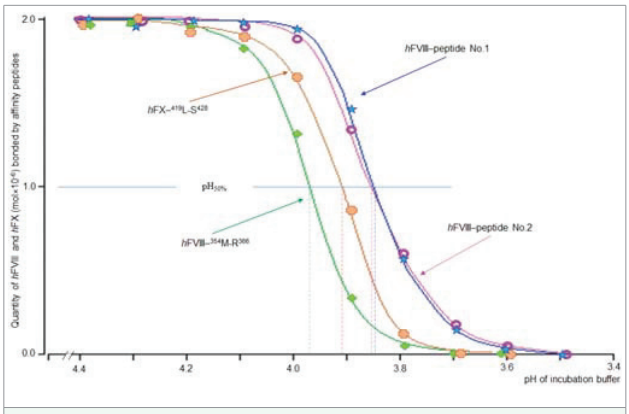

Figure 2: Binding *h*FVIII by peptides No.1, No.2, ^{354}M-R^{366} and binding *h*FX by peptide ^{419}L-S^{428}. Peptides were synthetized on membranes as described on chapter "Material and Methods".

For the development of the affinity ligand for FVIII, peptides No.1 and 2, revealed in the searching for a binding site on the FX, were accepted as a basis (basic peptides). Both peptides exhibited equal affinity for FVIII (Figure 1). A possibility to increase the affinity was considered as a creation of a positive charged center in peptide No. 2 by the ^{5}S/R mutation, as well as an increase of the negative charge of the position 12 due to the ^{12}L/F mutation. Both mutations should lead to strong electrostatic interaction with the FVIII sequence (Table 2, peptides No. 3-8).

^{11}I/P mutation (HPI_I=+3.1, pI_I=5.9; HPI_P= -0.3, pI_P=6.5) also looks promising due to negative charge going to zero and resulting light hydrophilicity allowing interaction with the hydrophobicity of tyrosine at position 11 (HPI_Y= +0.1, pI_Y=5.7) in the FVIII sequence (Table 3, No.5,7,8). Similar reasoning suggested S/G mutation in the position 9 (HPI_S= -3.1, pI_S=5.7; HPI_G=+0.7, pI_G=6.0), where the interaction of protein's R with peptide's S (HPI_R= -7.5, pI_R=10.8) according to charge only, seems to be rather weak. In contrast to the peptide's ^{9}G, ^{9}S due to its higher MW, will improve peptide linearity and hydropathy, and will interact with protein's R electrostatically.

As our experiments showed, further mutations can lead to an increase in the affinity of peptides No.1-2 for FVIII, however, there is a risk to sharply reduce the specificity of the peptide to the protein due to the loss of genetically inherent individuality at the sequence. Four out of fifteen of developed peptides (№ 7,8,14,15) had more than satisfactory affinity for FVIII (the difference from the base peptides was statistically significant, p<0.01), the best peptides were chosen and synthesized for use as ligands in chromatographic studies.

Having in hand identified FVIII binding site on FX, we calculated the antisense, i.e. primary affinity peptide for the FX-binding site (Table 4). Two α-, one β- and two γ-skeletal aa_s explicitly determined seven aa_s in antisense peptide. Other seven were chosen from aa pairs determined by basic and invariant aa_s of the sense FX-derived ^{354}M-R^{366} peptide (Table 3). At the direction 3'→5' two challengers on antisense peptide for positions 2, 5, 8, 9, 11 and 13, at the direction 5'→3' – two for position 11, three for position 7 and four for positions 2, 5, 6, 8, 9, 10, 12 and 13 were determined.

^{2,5}C had just one and minimal advantage over ^{2,5}W – an equal MW with sense threonine (MW_T=119; MW_C=121; MW_W=204). But aa molecular weight doesn't have a tangible effect on the length of the peptide chain, rather on its linearity [50]. Minor negative charge of

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Table 2: The peptide array library for the development high affinity peptides to *h*FVIII on the basis of calculated peptides No.1 and 2.

Peptide No.	Sense peptide in the *h*FVIII sequence													Affinity to *h*FVIII by $pH_{-50\%}$ (M±SEM, n=5)
	P	L	V	L	A	P	D	D	R	S	Y	K	S	
	Amino acid sequence of the calculated antisense peptides, synthetized on membrane (amino acid position number in the peptide)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	
1	**G**	**N**	**Q**	**N**	**R**	**G**	**L**	**L**	**S**	**R**	**M**	**F**	**R**	3.86±0.09
2	**G**	**Q**	**H**	**K**	**S**	**G**	**I**	**V**	**S**	**R**	**I**	**L**	**R**	3.85±0.06
3	G	Q	H	K	**R**	G	I	V	S	R	I	L	R	3.73±0.07
4	G	Q	H	K	S	G	I	V	S	R	I	**F**	R	3.82±0.09
5	G	Q	H	K	S	G	I	V	S	R	**P**	L	R	3.78±0.11
6	G	Q	H	K	**R**	G	I	V	S	R	I	**F**	R	3.70±0.12
7	G	Q	H	K	**R**	G	I	V	S	R	**P**	L	R	3.66±0.05
8	G	Q	H	K	**R**	G	I	V	S	R	**P**	**F**	R	3.51±0.06
9	G	Q	H	K	S	G	I	V	**G**	R	I	L	R	3.71±0.08
10	G	Q	H	K	**R**	G	I	V	**G**	R	I	L	R	3.74±0.10
11	G	Q	H	K	S	G	I	V	**G**	R	I	**F**	R	3.81±0.05
12	G	Q	H	K	S	G	I	V	**G**	R	**P**	L	R	3.76±0.08
13	G	Q	H	K	**R**	G	I	V	**G**	R	I	**F**	R	3.71±0.11
14	G	Q	H	K	**R**	G	I	V	**G**	R	**P**	L	R	3.62±0.09
15	G	Q	H	K	**R**	G	I	V	**G**	R	**P**	**F**	R	3.46±0.06

Note 1. Red letters – *h*FVIII-derived peptide ^{416}P-S^{428}, yellow box with blue letters – mutations of the basic peptides
Abbreviations: *h*FVIII: Human Factor VIII

Table 3: The calculation primary affinity peptide to *h*FX-binding site.

	Recognized *h*FX-binding site (sense peptide)												
Reading direction	**M**	**T**	**Q**	**K**	**T**	**G**	**I**	**V**	**S**	**G**	**F**	**G**	**R**
	Antisense peptides												
	Y	**C**	**V**	**F**	**C**	**P**	**Y**	**H**	**R**	**P**	**K**	**P**	**A**
3'→5'		**W**			**W**			**Q**	**S**		**N**		**S**
5'→3'	**H**	**C**	**L**	**L**	**C**	**P**	**Y**	**H**	**R**	**P**	**K**	**P**	**A**
		G			**G**	**T**	**N**	**N**	**T**	**T**	**E**	**T**	**S**
		R			**R**	**S**	**D**	**D**	**G**	**S**		**S**	**P**
		S			**S**	**A**		**Y**	**A**	**A**		**A**	**T**
aa position No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Primary 3'→5' peptide No.16	**Y**	**W**	**V**	**L**	**W**	**P**	**Y**	**H**	**R**	**P**	**K**	**P**	**A**
Primary 5'→3' peptide No. 17	**H**	**C**	**L**	**L**	**C**	**P**	**N**	**H**	**R**	**P**	**K**	**P**	**A**

Abbreviations: *h*FX: Human Factor X; aa: Amino Acid

^{2,5}W will not interfere with its hydropathic interaction with T which also has an insignificant negative charge (HPI_W=+1.5, pI_W=6.0; HPI_T= −0.8, pI_T=5.9; HPI_C= +0.2, pI_C=5.1). Therefore, our selection was stopped at tryptophan as basis aa at positions 2 and 5. Nevertheless, the mutation W/C was not discarded and kept for practical testing (Table 4).

Positively charged ^{8}H, ^{9}R and ^{11}K in the antisense peptide got the advantage over Q, S and N due to histidine, arginine and lysine will interact with sense peptide's valine, serine and phenylalanine, respectively, not by hydropathy properties only, but electrostatically also according to positive and negative charges. The peptide's alanine (HPI_A=+1.0, pI_A=6.0) should occupy position 13 to complement sense arginine (HPI_R= −7.5, pI_R=10.8) in the sequence of FX because of the same two interactions: by hydropathy and electrostatic attraction. A weak negative charge of serine, most likely, wouldn't be able to compensate the hydropathic antagonism with arginine, which would reduce the affinity of the peptide to FX. For a possible increase of peptides affinity to FX, one should also take into consideration an increase of positive charge at positions 1, 7, and 11, introducing the corresponding mutations ^{1,7}H/K or ^{1,7}H/R and ^{11}K/R (peptides No. 32-35, Table 4).

Both peptide array libraries comprised 15 and 35 peptides only. Moreover, already in the initial stages of construction, it was clear that the peptides will have a sufficiently high affinity for factors VIII and X to be used as ligands in affinity chromatography.

6.4 Peptide's affinity establishing to FVIII and FX

The affinity of peptides, measured by the 50% level of dissociation of the corresponding protein from the peptide synthesized on membrane ($pH_{50\%}$), is presented in Tables 2 and 4. All peptides of both array libraries showed high affinity for their proteins (close to the level of Protein A and G affinity for IgG [49]). According to our results, the affinity level corresponding $pH_{50\%}$=3.45÷3.47 will show complete dissociation at pH<2.5, which can lead to the protein

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Table 4: The peptide array library for the development high affinity peptides to *h*FX on the basis of calculated peptides No.1 and 2.

Peptide No.	Sense peptide at the *h*FX sequence													Affinity to *h*FX by $pH_{-50\%}$ (M±SEM, n=5)
	M	T	Q	K	T	G	I	V	S	G	F	G	R	
	Amino acid sequence of the calculated antisense peptides, synthetized on membrane (amino acid position number at the peptide)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	
16	**Y**	**W**	**V**	**L**	**W**	**P**	**Y**	**H**	**R**	**P**	**K**	**P**	**A**	3.77±0.05
17	**H**	**C**	**L**	**L**	**C**	**P**	**N**	**H**	**R**	**P**	**K**	**P**	**A**	3.68±0.05
18	**H**	W	V	L	W	P	Y	H	R	P	K	P	A	3.71±0.08
19	Y	W	V	L	W	P	**H**	H	R	P	K	P	A	3.73±0.04
20	**H**	W	V	L	W	P	**H**	H	R	P	K	P	A	3.51±0.10
21	Y	**C**	V	L	W	P	Y	H	R	P	K	P	A	3.73±0.07
22	Y	W	V	L	**C**	P	Y	H	R	P	K	P	A	3.76±0.09
23	Y	**C**	V	L	**C**	P	Y	H	R	P	K	P	A	3.74±0.06
24	**H**	**C**	V	L	W	P	Y	H	R	P	K	P	A	3.66±0.11
25	**H**	W	V	L	**C**	P	Y	H	R	P	K	P	A	3.70±0.06
26	**H**	**C**	V	L	**C**	P	Y	H	R	P	K	P	A	3.61±0.06
27	Y	**C**	V	L	W	P	**H**	H	R	P	K	P	A	3.76±0.12
28	Y	W	V	L	**C**	P	**H**	H	R	P	K	P	A	3.70±0.05
29	Y	**C**	V	L	**C**	P	**H**	H	R	P	K	P	A	3.58±0.08
30	**H**	**C**	V	L	**C**	P	**H**	H	R	P	K	P	A	3.54±0.09
31	**H**	W	V	L	W	P	**H**	H	R	P	**R**	P	A	3.47±0.11
32	**K**	W	V	L	W	P	Y	H	R	P	**R**	P	A	3.43±0.08
33	**K**	W	V	L	W	P	**K**	H	R	P	**R**	P	A	3.40±0.12
34	**R**	W	V	L	W	P	Y	H	R	P	**R**	P	A	3.40±0.07
35	**R**	W	V	L	W	P	**R**	H	R	P	**R**	P	A	3.37±0.10

Note 1. Red letters – *h*FX-derived peptide ^{354}M-R^{366}, yellow box with magenta letters – mutations of the primary peptides
Abbreviations: *h*FX: human Factor X

denaturation, especially if protein has a high MW [51]. Therefore, peptides No. 15 (Table 2) and 31-35 (Table 4) were discarded and not considered as ligands for affinity chromatography.

As suggested above, the introduction/enhancement of a positive charge at positions 5 (peptides for FVIII, Table 2), 1, 7, and 11 (peptides for FX, Table 4) led to a statistically significant increase of the peptide affinity (p<0.01 for most of cases, rarely p<0.001). Peptide affinity was also slightly increased by mutations in favor of hydropathic and electrostatic peptide-protein interaction (^{5}R/S, ^{9}S/G, Table 2) or in favor of hydropathic properties with the same weak aa charges (^{11}I/P, Table 2). The affinity of the peptide minimally depended on the aa MW (^{12}L/F, Table 2; ^{2,5}W/C, Table 4). Apart from mutations introducing aa_s with a high positive charge, each individual indicator (hydropathic properties, negative charge, MW) did not lead to statistically significant changes of affinity (p>0.1), but showed a trend. Outlined in tables mutations changed the peptide's potential to interact with the protein statistically significantly (p<0.01).

Chromatographic gel with peptide affinity ligands synthesis and verification of its dynamic binding capacity and specificity

Every chromatographic specialist knows well that good affinity gel has to have a high specificity and high Dynamic Binding Capacity (DBC) for target protein [42]. In our case, it was desirable to obtain after first affinity chromatographic step FVIII or FX from human plasma with purity at least 85% and with minimal impurities of proteins with similar properties.

For the synthesis FVIII-affinity gel we chose peptides No.8 – GQHKRGIVSRPFR (SRF) and No. 15 – GQHKRGIVGRPFR (GRF). For purification of FX from human plasma peptides No. 30 – HCVLCPHHRPKPA (CCK) and No. 31 – HWVLWPHHRPRPA (WWR) were chosen. After peptides "sealing" the following chromatographic gels were obtained in the 5 ml columns BabyBio: SRF-WorkBeads 40/1000 with a peptide density 19.3 mg×mL^{-1} (~20.8 μmol×mL^{-1}), GRF-WorkBeads 40/1000 (a peptide density 20.8 mg×mL^{-1}, ~21.4 μmol×mL^{-1}), CCK-WorkBeads 40/1000 (a peptide density 16.5 mg×mL^{-1}, ~22.5 μmol×mL^{-1}), WWR-WorkBeads 40/1000 (a peptide density 18.3 mg×mL^{-1}, ~19.7 μmol×mL^{-1}).

The DBC with enough residence time 10 min for both FX affinity gels was around 48-50 mg×mL^{-1}. The enough residence time for coupling FVIII for CCK-WorkBeads was 15-17 min while for WWR-WorkBeads 8 min only. Th e DBC for these gels was diff erent too: CCK-WorkBeads – 29 mg×mL^{-1}, WWR-WorkBeads – 48 mg×mL^{-1}.

The chromatogram of the affinity purification of FX from donor blood plasma using 5 ml column BabyBio GRF-WorkBeads 40/1000, as well as the process of checking the column life-time for 1-250 complete chromatographic cycles (equilibration, plasma application, washing 1, washing 2, elution, regeneration and sanitation: full volume – 33 Vc) are presented in Figure 3.

It was shown that chromatographic fractions 1 and 2 did not contain FX, whereas in fraction 3 a total 7.692 mg, and in fraction 4 – 0.206 mg FX was determined, i.e. 91.4 and 2.5% respectively of the total 8.414 mg of FX contained in 1000 mL of plasma, which was used to obtain an enriched fraction of clotting factors on AEX Sepharose FF (GE Healthcare AB, Sweden) [43]. In fraction 2 0.463 mg of total protein were determined, which was non-specifically bound proteins (100% dissociation at 200 mM NaCl in equilibration buffer) that did not contain coagulation factors. Fraction 4 is consisted almost of completely denatured proteins with traces of FX. According to electrophoresis under nonreduction conditions (Figure. 3b) one main band with a molecular weight in the range of 55-57 kDa was determined in the

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chromatographic fraction 3, which corresponds to the native molecule FX (Figure. 3b, track 3). Further FX on the electrophoregram was identified by the rabbit polyclonal anti-hFX antibodies conjugated with fluorescein. A traces of FX impurities were detected in the 14-20 kDa zone (Figure. 3b, track 2), which did not contained FIX or FVIII fragments. The purity of the main protein was 92.7%. Using SRF-WorkBeads 40/1000, almost the same results were obtained: FX yield was 88.7%, purity – 89.1% without FIX impurities or FVIII fragments (statistically insignificant difference, in both cases $p>0.1$).

The plasma derived FVIII was purified on the BabyBio column packed with 5 ml WWR- or CCK-WorkBeads 40/1000 (chromatograms not shown). The yield from total FVIII content (4.361±0.283 mg) in the enriched fraction [37] was the following: 93.7% and 90.3%, respectively. The SDS-PAAG test purity not shown.

Further studies showed lower absolute percentage of FVIII content (41.7-46.8) after purification on the affinity gels WWR- or CCK-WorkBeads 40/1000. It was found by ELISA testing that the collected fraction contains FVIII, VWF and a small part of other proteins impurities. Data is presented in the Table 5.

It is well known that maintenance of normal levels of FVIII is dependent on its formation of a complex with VWF. FVIII circulates as an inactive pro-cofactor in complex with VWF [52]. In our case, for testing affinity gels-based FVIII purification we have used a high molecular weight protein fraction from blood plasma, specially processed for a more complete formation of the FVIII-VWF complex [37]. Therefore, an equimolar complex of factors was isolated with a high purity of 85.5-91.7%, which is satisfactory for affinity chromatography [53].

Peptide-affinity chromatographic gel life-time

To evaluate the stability of affinity peptide gels columns the BabyBio GRF-WorkBeads 40/1000 was chosen. All chromatographic manipulations were performed according "Peptide-affinity chromatographic gel life-time determination" chapter and using an ÄKTAexplorer with integrated UNICORN soft w are version 5.01. A new and unused column was employed for the study and was conditioned by performing ten injections of enriched fraction of clotting factors obtained after AEX Sepharose FF [43] captured from low molecular protein fraction [37] of human plasma until a constant peak 3 area and peak 3 height (Figure. 3a) were observed. Over the course of the study standard and excellent stable resolution of eluted non-specifically bound proteins (peak 2) and denatured proteins (peak 4) from the main FX (peak 3) was observed as shown in Figure 3a and 3c.

As Figure 3c shows, consistent retention times were observed for the main peak over the course of 250 injections, with a retention time difference of just 0.062 Vc between injection numbers 25 and 250; this illustrates the absence of chemical or electrostatic interactions with the column stationary phase following extensive use. Similarly, excellent peak symmetry and stable configuration between injection numbers 1 and 250 were detected throughout the column lifetime stability study, further demonstrating a lack of secondary interactions with the column packing material and hardware across the lifetime of the column.

Attempts to identify an affinity peptide that can leaching from the chromatographic matrix by determining tryptophan fluorescence in

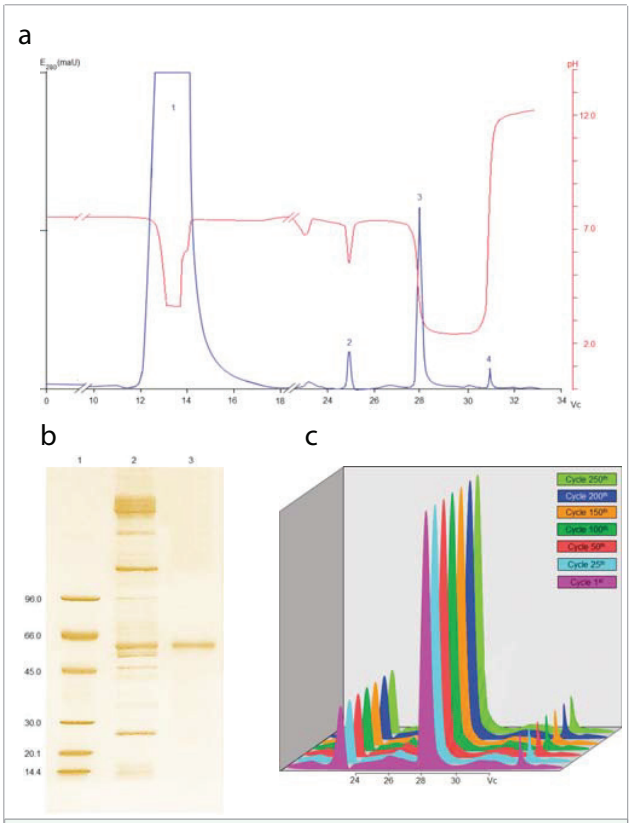

Figure 3: (**a**) The chromatogram of the affi nity purifi cation of FX from donor plasma on column BabyBio with 5 mL GRF-WorkBeads 40/1000: 1 – unbound proteins fraction, 2 – unspecifi ed bound proteins, washed by buff er with 200 mM NaCl, 3 – affi nity bound proteins, eluted by 20 mM citric acid-Na_2HPO_4 buff er, pH 2.6; 4 – denatured proteins washed by 0.05 M NaOH.
(b) SDS-PAGE plate (4-20%) developed under nonreducing conditions and stained by $AgNO_3$: 1. MW standards, 2. Fraction 2 from column BabyBio with 5 mL of GRF-WorkBeads 40/1000, 3. Fraction 3 included FX from column BabyBio with 5 mL of CRF-WorkBeads 40/1000.
(c) Checking the GRF-WorkBeads 40/1000 chromatography gel life-time during 1-250 complete chromatographic cycles. All process was carried out as described in the chapter "Materials and methods"..

Table 5: Proteins content in major chromatographic fractions collected from *h*FVIII affinity gels.

Proteins	Protein content in collected chromatographic fractions (M±SEM, n=5) from			
	WWR-WorkBeads		CCK-WorkBeads	
	mg	%	mg	%
*h*FVIII	4.086±0.361	46.8	3.938±0.522	41.7
*h*VWF	3.920±0.417	44.9	4.136±0.380	43.8
Other proteins	0.725±0.105	8.3	1.369±0.094	14.5

Abbreviations: *h*FVIII: Human Factor VIII; *h*VWF: Human Von Willebrand Factor

eluates, washing/cleaning buffers have been unsuccessful. Also, the attempt to determine the possible leaching of the peptide ligand by the difference in its density on the new and used in 250 cycles of chromatographic gels was not statistically significant. Thus, the above results clearly indicated that the dynamic capacity of the synthesized affinity gel with a peptide ligand GRF-WorkBeads 40/1000 was remained stable for at least 250 chromatographic cycles.

Thus, 35 high affinity peptides were developed during 12 hours of literature investigations and calculations by one person. Another 72

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hours were spent for peptide's libraries synthesis and experimental determinations of the affinity level of the calculated peptides. We really spend more time and efforts on discussing results and writing this manuscript.

CONCLUSION

Of course, we do not forget that serious preliminary work for our developments was done by the authors who have found that certain amino acid sequence of FVIII interact with FX, i.e. it is affine for site on FX [48]. On the other hand, a huge amount of research on protein-protein or protein-peptide interactions has been accumulated today, for example, for coagulation factors [54-56]. Moreover, often the results of these studies are not focused on the development of affine chromatographic gels. The proposed approach translates the results of these studies into a practical field.

Reducing the time and costs for the development of high affinity peptides will inevitably lead to a decrease in the cost of affinity chromatographic gels, the widespread use of which in technological processes will reduce the number of stages of purification of target proteins and, naturally, reduce the cost of pharmaceuticals.

However, having high affinity and, most importantly, specific peptides for the proteins, it would be a big mistake to limit ourselves to creating chromatographic gels only. For example, peptides No. 15 (Table 3) and 31-35 (Table 5) were discarded and not considered as ligands for affinity chromatography due to their very high affinity for clotting factors. But these peptides can be good candidates as ligands in the test systems for factors FVIII and X. This will be a valid accurate and important aim of our next study. Science, pharmaceutical industry and medicine need high-quality test systems, inhibitors and activators of enzymes, blockers of bacteria and viruses or receptors through which they enter the cell. High-affinity and specific low molecular weight peptides, which, by non-injection route, easily penetrate an intestinal wall and enter blood stream, claim all this and much more.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest Statement

Authors declare no conflict of interest. The study was performed at a time when authors were employees of Neutromics Ukraine TOV and working together at the biotechnology pilot plant in Raining (Boroo) Valley, Mongolia.

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by all authors too. The first draft of the manuscript was written by Serhiy P. Havryliuk and Heorgii L. Volkov and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

ETHICAL APPROVAL

This article does not contain any studies with human participation or animal performed by any of the authors.

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