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Proteomic and *In Silico* Analyses of Dialyzable Crocodile Leukocyte Extract: Identification of Putative Bioactive Peptides and Its Potential Beneficial Impact on Health

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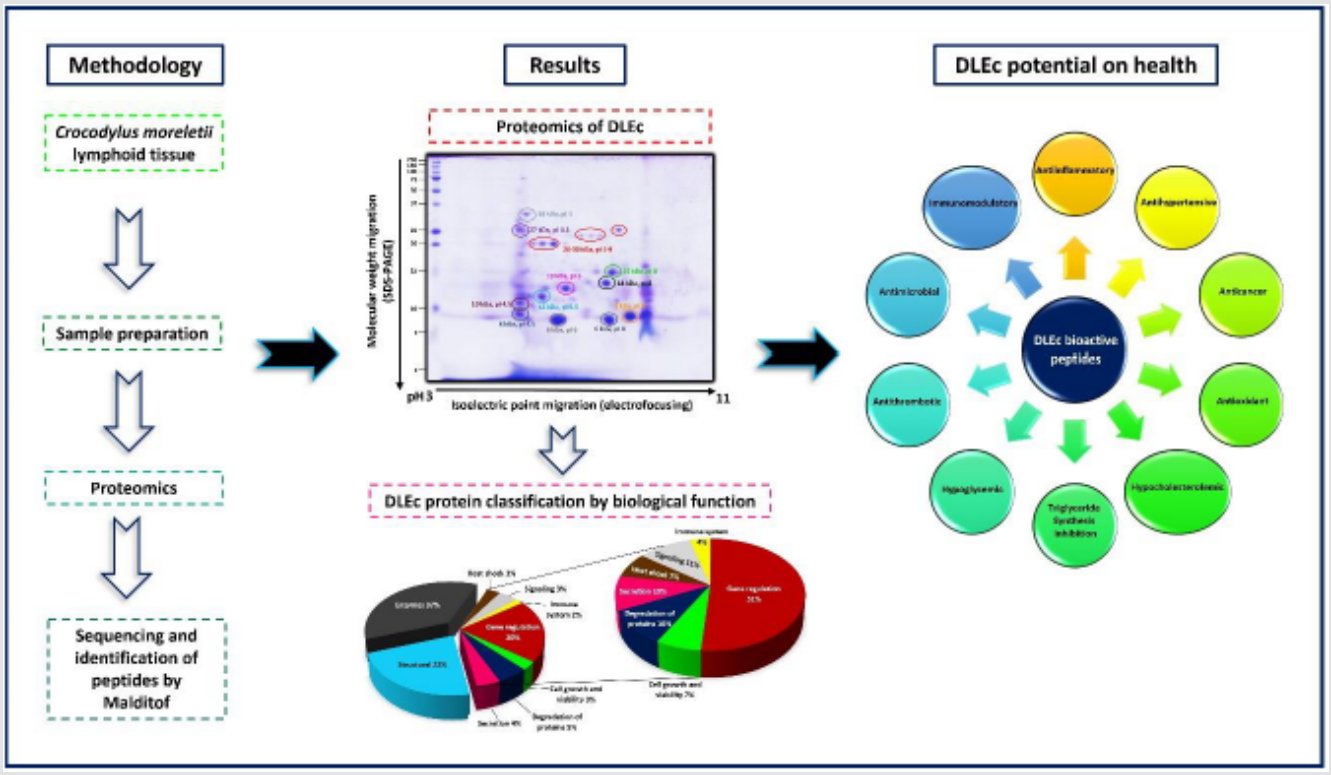
ABSTRACT

Currently, there is a great demand for effective, low-cost, and fewer side effects medicines due to the growing number of diseases in developing countries. The identification of bioactive peptides in compounds of natural sources or those generated artificially is a viable alternative that should be systematically investigated, particularly in those products that have shown immune modulating activity. Dialyzable leukocyte extract (DLE) from human or various species, a low molecular weight peptide fraction of immune cells, has been used for several decades and has clinically demonstrated its usefulness in various inflammatory diseases and those in which immune system functions are out of balance. However, due to its complexity and lack of characterization of its components, it has been difficult to determine its mechanisms of action. In the present investigation we performed proteomic, *in silico* and interactomic analyses of DLE obtained from *Crocodylus moreletii* lymphoid tissue (DLEc). The 2D proteomic characterization showed a heterogeneity of peptides in a molecular weight range of 38 to 6 kDa with pI of 4-9; by nanoLC-MS/MS orbitrap analysis it was demonstrated that it is constituted by 302 peptides, which we grouped into 5 main clusters; I. Regulators of gene expression, II. signaling, III. involved in secretion, IV. degradation pathway, V. heat shock, and to a lesser extent, immune system proteins. *In silico* we identified in its sequence several bioactive peptides, whose activity has been experimentally demonstrated in diverse study models. We found peptides with immunomodulatory; anti-inflammatory; phagocytosis-inducing; antihypertensive; anticancer; antioxidant; modulators of various metabolic pathways; antimicrobial and antithrombotic activities. Our analyses suggest that DLEc is a heterogeneous mixture of peptide sequences containing bioactive peptides with enormous potential as pharmacological adjuvants in different pathologies.

Keywords: Dialyzable Leukocyte Extract; Bioactive Peptides; Health Impact; Proteomics; Bioinformatics

Abbreviations: DLE: Dialyzable Leukocyte Extract; DTT: Dithiothreitol; IEF: Isoelectric Focusing; SDS: Sodium Dodecyl Sulfate; LTQ: Linear Trap Orbitrap Velos

Graphical Abstract



Graphical Abstract.

Introduction

Immunotherapy, also called biological therapy, is a type of treatment that stimulates the body's natural defenses to combat various complex diseases in which the immune system is compromised or in those that require the regulation and proper functioning of the immune system. Substances produced by the body or manufactured in a laboratory are used to restore, stimulate, or replenish immune function in cancer, infections, or other degenerative diseases, as well as to lessen the side effects of the treatments used. The objective can be prophylactic (prevention) or therapeutic (curative or maintenance). Immunotherapy includes the use of antibodies, vaccines, growth factors, compounds extracted from plants and of relevance the dialyzable leucocyte extract (DLE), "erroneously called transfer factor". DLE is a complex mixture of bioactive peptides obtained from human peripheral blood leukocytes or lymphoid tissues of various species. Clinical practice and various research studies suggest that DLE is a safe and effective treatment option for a wide range of pathologies. However, due to the broad spectrum of biological activity, resulting from the complexity and diversity of peptides it contains, the study of the mechanisms of action is complex and, in many cases, its probable action is not sufficiently documented and investigated. DLEs have been developed in several countries as supplements to treat immune-related diseases (Ojeda, et al. [1-3]); Inflammatory diseases (Hernández, et al. [4]); infectious diseases (Cabezas, et al. [5-8]); in asthma (Cabezas, et al. [9]); as an adjuvant in cancer treatment (Pineda, et al. [10-14]). On the other hand, our research group, has focused on, addition to studying the inflammatory effects in different disease models, such as osteoarthritis (Acosta, et al. [15]); prostatitis (Pérez-Alvarado, et al. [16]); cervicitis in premalignant lesions associated with human papillomavirus (Acosta, et al. [17]); and influenza (Pérez, et al. [18]); in determining the mechanisms of action in cellular and animal models. Research suggests that modulation of innate immunity and regulation of the inflammatory process constitute the main mechanisms exerted. Further systematized research on i. The exact composition; ii. Gastrointestinal hydrolysis and release of specific bioactive peptides; and iii. The mechanisms of action and bioavailability of the peptides that compose it, will allow a better prescription for specific pathologies, as well as for the development of new drugs and novel and specific therapies that contribute to current treatments. In this work we performed the proteomic characterization of DLEc by 2D and nanoLC-MS/MS orbitrap analyses, we identified bioactive peptides that can interact and modulate different molecular pathways within the cell related to immunomodulatory and anti-inflammatory effects. Our analyses suggest that DLEc is a heterogeneous mixture of peptide sequences containing bioactive

peptides with enormous potential as pharmacological adjuvants in different pathologies.

Materials and Methods

Protein Preparation

Pellets of trichloroacetic acid-precipitated proteins were washed four times in a large volume of 96% ethanol and then resuspended by scraping and extensive pipetting in 5 ml of an isoelectric focusing (IEF) sample solution composed of 8 M urea, 4% (wt/vol) 3-[(3-cholamidopropyl) dimethyl ammonio]-1-propanesulfonate (CHAPS), 40 mM Tris, 2% dithiothreitol (DTT), and 0.2 (wt/vol) Bio-Lyte 3/10 (Bio-Rad). The clear solution contained approximately 0.3 mg/ml protein.

2-D Electrophoresis Separation of Proteins and Spot Quantitation

DLE protein mixture was resolved first by IEF on pH 3 to 10 (nonlinear) ready-made, 13-cm, immobilized pH gradient strips (Immobiline DryStrips; GE Health care), applied to an Ettan IPGphor III (AmershamBiosciences). IEF was carried out at 500 V for 1 h, followed by two gradients of 1000 V; for 1 h and 2.5 h; a final step at 8000 V for 25 and 55 min. Strips were then processed for the second-dimension separation by a 10-min incubation in 6 M urea, 2% sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% (wt/vol) DTT, followed by a 10-min incubation in a similar solution in which the DTT was replaced by 2% iodoacetamide. Strips were applied to 12.5% SDS-polyacrylamide gels, and electrophoresis was conducted at 100 V for 7 h. Gels were stained with Coomassie blue G-250 (Bio-safe Coomassie; Bio-Rad) and spots detected and analyzed. At least three independently obtained DEL biological sample were evaluated by 2-D electrophoresis, at least three gels were used. The gels from which the spots were excised for sequencing were stained with colloidal Coomassie blue for 1 h, after fixation with 30% ethanol, 5% glacial acetic acid for 1 h. Finally, the gels were washed three times with water and then washed with water. Finally, three washes were made with milliQ water to distain and visualize the spots.

Sample Preparation for Malditof Sequencing

Precipitation: The lyophilized sample of approximately 50 mg of DLEc was resuspended in 100 µl of 8 M urea, precipitated with chloroform-methanol and resuspended again in 50 µl of 8M urea for digestion. Digestion with trypsin: 50 µg of the precipitated sample was taken. Added 10 mM DTT (final concentration) and incubated for 1 h at 37ºC. Iodoacetamide was added to a final concentration of 55mM, incubated at RT 30 min in the dark. Diluted with ammonium bicarbonate to 2M urea (final concentration). Recombinant trypsin (Roche) was added at a ratio of 1:20 with the protein and incubated O/N at 37 C.

Desalting and Quantification of Protein Concentration: Sample clean-up was performed using a PorosR2-type resin@ and peptides were eluted with 80% acetonitrile (ACN) in 0.1% TFA. Samples were lyophilized in Speed-vac and dissolved in 50 µl of 2% formic acid.

Analysis by NanoLC-MS/MS (Orbitrap): Peptides were separated on-line with an Easy-nLC (Proxeon) using a 160 x 75 µm NS-AC-11-dp3 C18 column (BioSphere) and a Pre-Column Easy-column C18-A1 (Proxeon) at a flow rate of 250 nL/min using a 245 min gradient: 190 min 2-30% of B phase, 40 min 30-40% of B phase 10 min 40-90% of B phase and 4 min 99% of B phase. The composition of solvent A was formic acid 0.1%, acetonitrile 2%, water 98% and solvent B was 99.9% acetonitrile with 0.1% formic acid. Peptides were analyzed using a linear trap Orbitrap Velos (LTQ Orbitrap Velos) hybrid mass spectrometer (Thermo Electron Corp., Bremen, Germany). The electrospray voltage of 1.7 kV versus the inlet of the mass spectrometer was used. The mass spectrometer was operated in the non-data-dependent mode. The parameters for ion scanning were the following: Full-scan MS (400-1800 m/z) plus top 15 peaks MS2.The scanning was performed using a dynamic exclusion list (30s exclusion list size of 500).

Protein Identity: For protein identification, the non-redundant Swissprot (20233 sequences) database was searched by a licensed version of MASCOT 2.3.0 using the Proteome Discoverer software version 1.2.0.208 (Thermo). Search parameters were oxidized methionine as variable modification, carbamidomethyl cysteine as fixed modification, peptide mass tolerance 10 ppm, 1 missed trypsin cleavage site, MS/MS fragments tolerance 0.8 Da. In all protein identification, the FDR was fixed at 1%.

***In Silico* Analyses:** Using STRING version 10.5 (https://version-10-5.string-db.org/) we obtained a general interactome of the identified DLEc proteins, which were grouped according to their biological functions. Similarly, proteins from DLEc with immune functions were challenged against the human proteome in STRING to identify putative interactions to date possible molecular mechanism. Predictions were made with values equal to or up to 70% probability in STRING. To evaluate the immunomodulatory effect of DLEc targeted *in silico* analysis was performed making an interactome using the DLEc proteins and IL2, IL18, IL1A, IL12A, IL12B, IFNG, CSF2, TNF, IL12RB1, IL12RB2, IL1A, IL1B, IL2, IL4, IL6, IL23A, IL23B, XBP1 and IRF-4. Similarly, modulatory effect of inflammation was evaluated by determining the interaction of DLEc proteins with proteins of the TNFR-NFkB pathway, using TRADD, TRAF2, IKBKB, NFKB1, TNF human proteins. To identify peptide sequences with beneficial biological activity for health, we searched the literature for reports of bioactive peptides with biological effects proven experimentally in cell lines, murine models or in human clinical studies. The selected bioactive peptides with anti-inflammatory;

phagocytosis-inducing; antihypertensive; anticancer; antioxidant; modulators of various metabolic pathways; antimicrobial and antithrombotic activities were identified in the amino acid sequences of DLEc proteins (Uniports, https://www.uniprot.org/).

Results

DLEc Proteomics

It has been postulated since its discovery that DLE, a leucocyte diafiltrate that passes through an exclusion pore of 5 kDa, is a mixture of small peptides bellow similar molecular weight. In this work, to define the identity of the constituent peptides of DLEc,2D electrophoresis analysis of the final product commercially called "Factran", was conducted in the Ethan II isoelectrofocusing equipment. (Figure 1) shows a Coomassie/silver-stained gel representative of the peptide components of the mixture. About 80 peptides were identified, mostly between 6 and 15 kDa, although there is also a group of proteins between 20 and 38 kDa, with a pI range from 4 to 9. The most evident spots were cut out of the gel and sequenced by nanoLC MS/MS. The identity of the peptides is presented in (Table 1). Cytoskeleton proteins, ribonucleoproteins, constituent peptides of energy metabolism enzymes, heat shock proteins, and interestingly, several ubiquitin constituent peptides were identified.

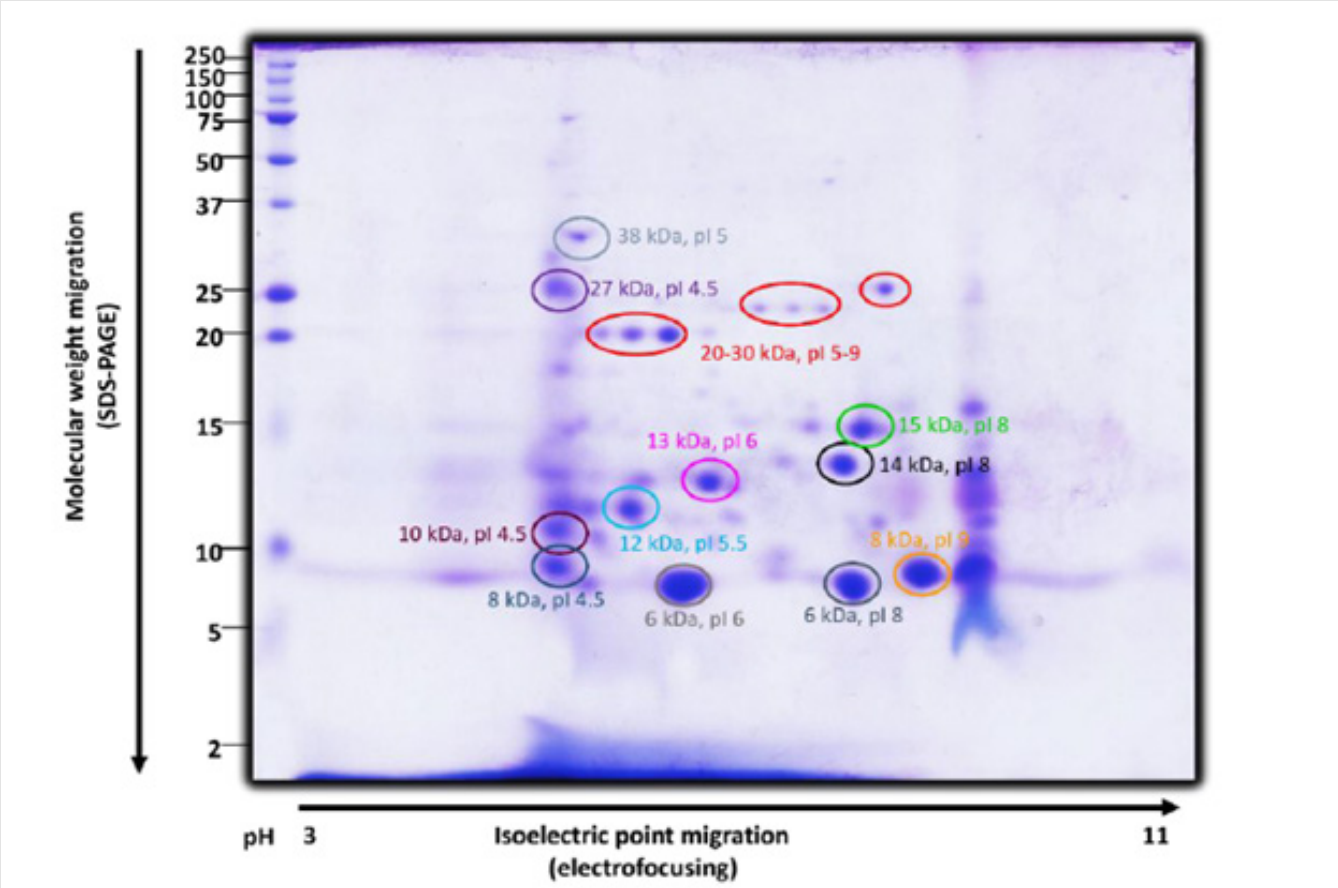


Figure 1: 2D proteomic analysis of DLEc. 400 µg of DLEc proteins were loaded and run on a 15% polyacrylamide gel. A protein profile with spots between 38 and 6 kDa is observed. The gel was stained with Coomassie blue. The spots indicated in the image were excised from the gel and sequenced by nanoLC-MS/MS (ORBITRAP). The identified proteins are summarized in Table 1.

Table 1: Protein sequence identity of 2D electrophoretic spots by Maldi tof and Madi tof-tof.

#Spot	Protein Sequence Identity
1	Chain A, Crystal Structure of the 35-36 8 Glycine Insertion Mutant of Ubiquitin
2	Unidentified
3	Unidentified
4	Unidentified
5	Inosine 5-monophosphate dehydrogenase
6	Unidentified
7	Unidentified
8	Unidentified
9	Rho GDP-dissociation inhibitor 1-like
10	Unidentified
11	Unidentified
12	Unidentified
13	Unidentified
14	Unidentified
15	mCG123153
16	Heterogeneous nuclear ribonucleoprotein H2
17	Unidentified
18	Unidentified
19	Ubiquitin, partial
20	Uncharacterized protein LOC100036820
21	Ubiquitin
22	Ubiquitin B, isoform CRA_e
23	c-src tyrosine kinase variant
24	Ribosomal protein S27a, isoform CRA d / ubiquitin B, isoform CRA_e
25	Hypothetical protein CENSYa_0233
26	Core binding factor beta
27	unnamed protein product
28	Unidentified
29	Ubiquitin, partial
30	Heterogeneous nuclear ribonucleoprotein B0b
31	Unidentified

DLEc Nanolc-MS/MS

Taking into consideration the high variety of peptides whose abundance is low; in parallel we made the complete peptide sequencing of DLEc, by nanoLC-MS/MS (ORBITRAP), in collaboration with the proteomic services unit of the Complutense University of Madrid. Approximately 302 constituent peptides of the DLEc were found by ion trap sequencing. As can be seen in the chromatogram, there is a significant heterogeneity in the relative abundance of the peptides obtained (Figure 2); bioinformatic analysis of these peptides was performed in the non-redundant Swissprot (20233 sequences) database using a licensed version of MASCOT 2.3.0 and the Proteome Discoverer software version 1.2.0.208 (Thermo). The identity of each peptide is show in Supplementary Table 1.

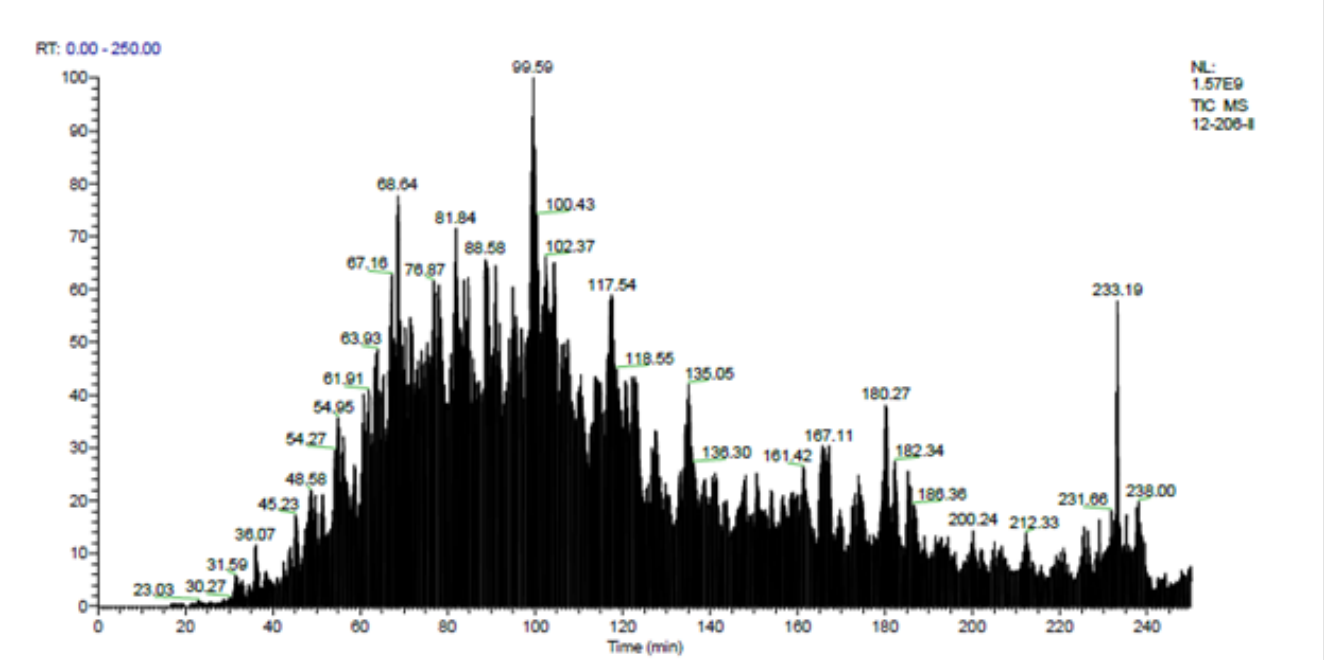


Figure 2: Chromatogram of DLEc performed with nanoLC-MS/MS (ORBITRAP). The heterogeneity and diversity of peptides generated as well as their relative abundance can be observed.

DLEc Bioinformatics Analysis

Identification and Classification of the DLEc Proteins: The bioinformatic analysis was performed based on the results obtained in 2D and total proteomics performed by nanoLC-MS/MS of the DLEc. By means of proteomics analysis we were able to identify the different peptides belonging to the proteins that make up the DLEc. The identified proteins were used to obtain different interactomes to propose molecular pathways that could potentially modulate different cellular events deregulated in pathological states. The result obtained shows that DLEc peptides match proteins involved in crucial processes for cell survival such as signaling, gene expression regulation, secretion, heat shock or response to diverse types of stress and protein degradation (Figure 3). Proteins were distributed in the different processes as follows: gene expression (51%); regulatory proteins (11%), degradation (10%), secretion (10%), heat shock (7%) and only 4% involved in the immune system (Figure 4). Proteins involved in immune functions were ITIH4, ANXA5, SERPINB1, SERPINB3 and C3. The interactome analysis of these five proteins, suggests that ITIH4, SERPINB1 and C3 can potentially interact each other; SERPINB3 only interact with C3, while ANXA5 did not interact with the any of them. The proteins were independently challenged on the platform with the human proteome to assess whether these DLEc proteins can interact with immune modulatory proteins (Figure 5). Interactomes showed that peptides can interact with human proteins involved in the immune system response (59%); in cell cycle or cell proliferation (23%); in apoptosis (15%) and, to a lesser extent, with master regulatory proteins (3%) (Figure 6). ANXA5 is able to interact and modulate IL2, IL6, IL10, IL7, CD34, IGBP1, TNF, TNFSF10, CD19, and CSF2 proteins; SERPIND1 interacts with C4A, C3 and IL6, 8; C3 interacts with CD64, CR1, CFI, CD55, CR2,CFD, ITGAM, CFHR3, 4, 5, C5AR1, 5, IL6, VSIG4, MPO, ELANE and CD19; SERPINB3 modulates SERPINB4, ELANE, GRN, AZU1, PRTN3, MP0, C3, LYZ and GCA proteins; and ITIH4 interacts with CCR5, CD34, LGALS3BP, CTSW, CD209 and IL6. Based on these analyses, the results suggest that peptides contained in DLEc may exert a modulatory effect on the immune system and other essential biological processes in the cell.

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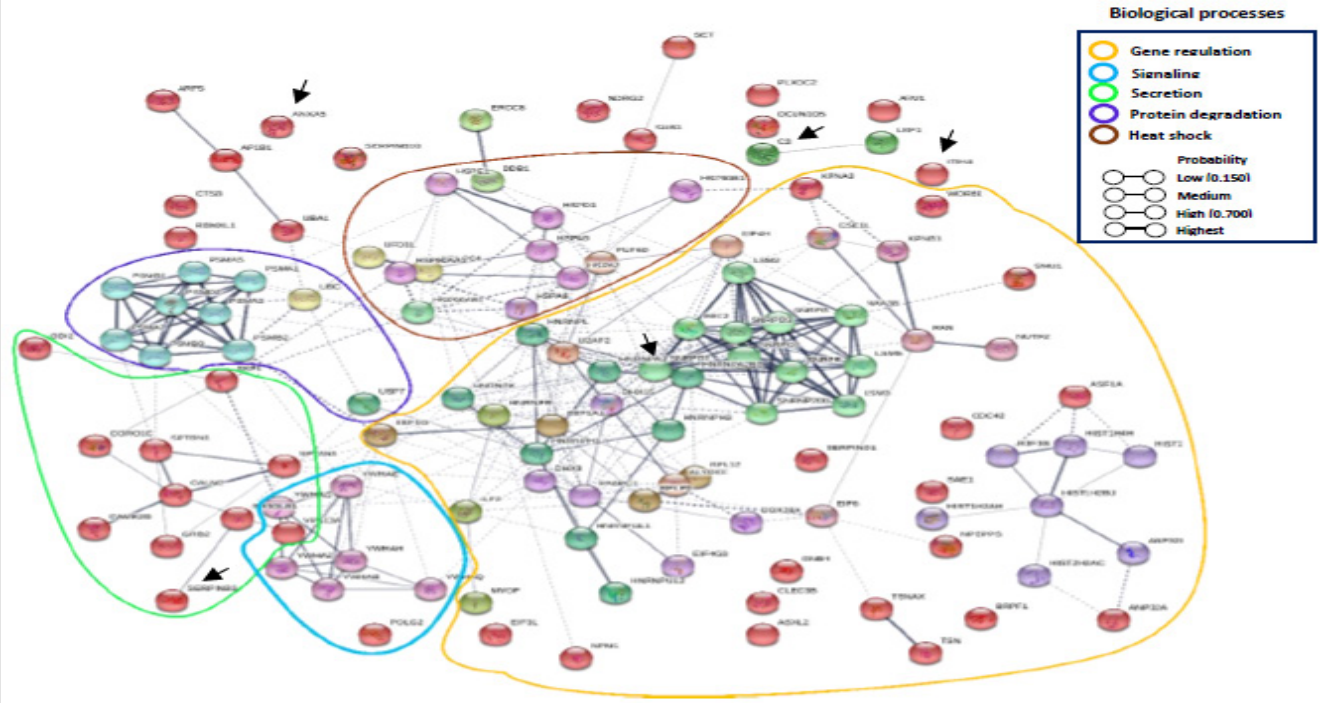

Figure 3: General interactome of DLEc. The clusters correspond to proteins with similar functions, arrows mark immune system related proteins.

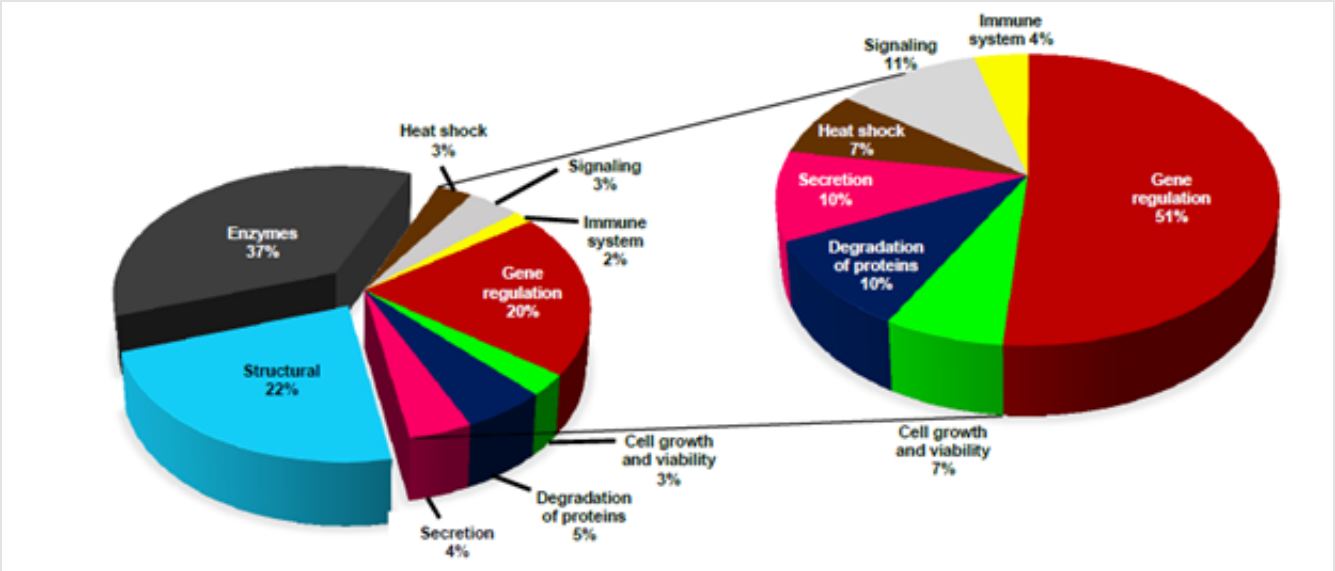

Figure 4: Distibution of DLEc identified peptides according to their function in different cellular processes.

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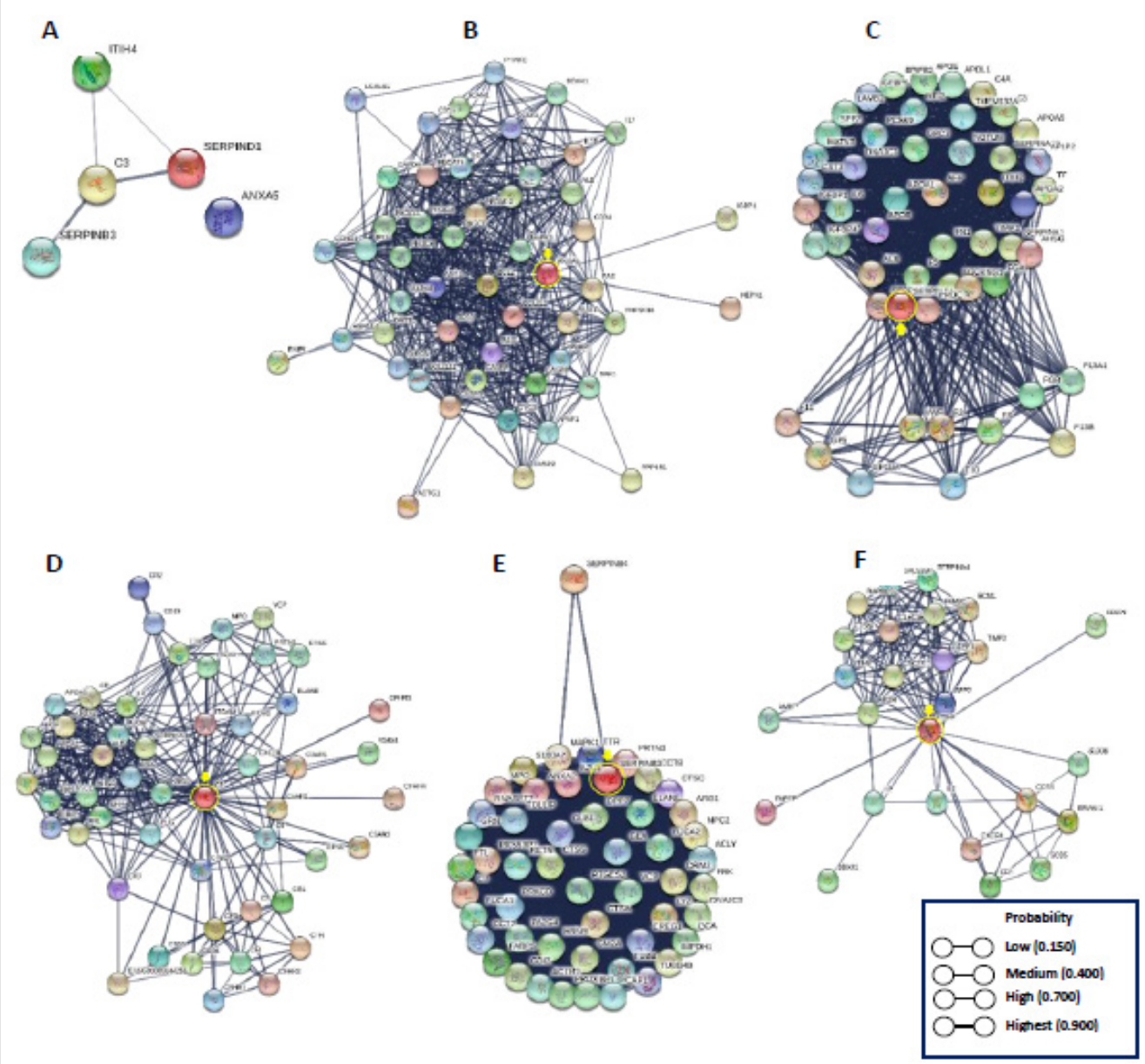

Figure 5: Interactomes between DLEc and human proteins.
A) Interaction of DLEc proteins that could modulate the immune response (ANXA5, SERPINB1, C3, SERPINB1 and ITIH4). Individual interactomes of each DLEc protein with the human proteome: B) ANXA5, C) SERPINB1, D) C3, E) SERPINB3 and F) ITIH4. Yellow circles and arrows indicate the DLEc protein.

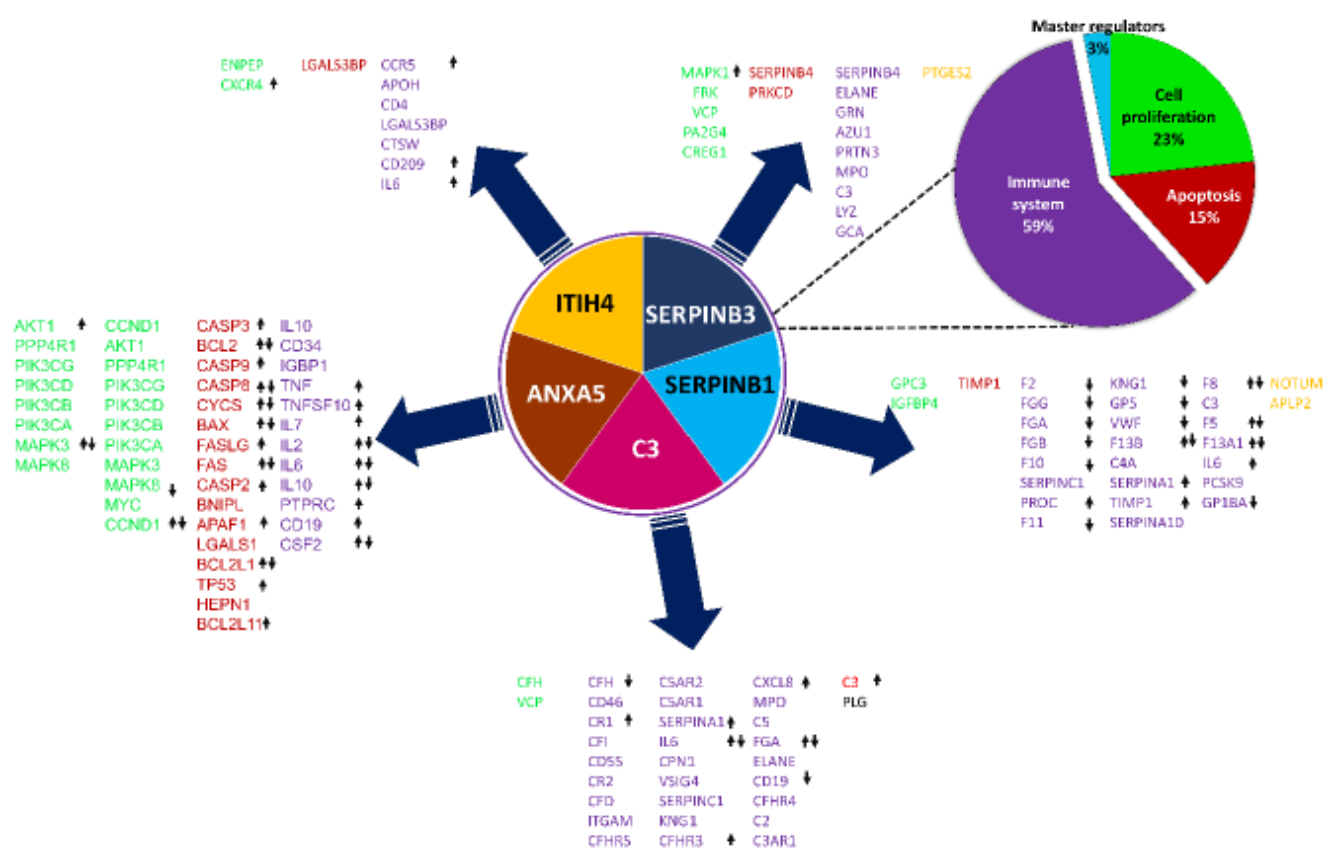

Figure 6: DLEc protein peptides and their possible participation as modulators. of different processes. Immune system (purple color); proliferation (green color); apoptosis (red color); and master regulators (blue color). The upward arrow indicates activation, the downward arrow indicates inhibition, and the proteins that present both, indicate that they can modulate activity in both directions, according to the cellular microenvironment.

Potential Health Impact of DLEc

DLEc as a Potential Regulator of Immune System: Interactomes evaluation could predict biological interactions between DLEc peptides and proteins involved in immune system modulation (Szklarczyk, et al. [19]). We found eight candidate proteins: C3, ANXA5, CAMK2G, ITIH4, PABPC1, GRB2, LRP1, and SERPIND1 that most likely regulate immune system cell activity by being involved in the modulation of the interleukins IL-1 α and β, IL- 2, IL-4, IL-6, IL-12 α and β, IL-18, IL-23, and the INF-γ factors GM-CSF or CSF2 2, TNF, XBP-1 and IRF-4 in mast and dendritic cells (Nicholas y Lesinski, et al. [20]); T cells and their differentiation to CD4+ and CD8+ T, macrophage activation, as well as in maturation and differentiation of B cells to plasma cells (Figure 7) (Kim, et al. [21]). The results obtained strongly suggest the function of DLEc as a potent immunomodulator of the immune system under pathological conditions, such as viral, bacterial, antifungal, or parasitic infections, cancer and immunological disorders in which bioactive peptides from different species has been evaluated in several studies (Fernández-Ortega, et al. [1,22-29]).

DLEc As a Potential Regulator of Inflammation: We also evaluated the possible interactions of DLEc proteins on NF-κB-mediated regulation of inflammation. The results obtained show that annexin 5, ITIH4, C3, CDC42 and LRP1 proteins from DLEc interact with TNF-α factor, likewise, CALM3 and YWHAB, from DLEc showed interaction with IKB1, whereas DHX9, ILF2, YWHAE, ILF2, and RAN from DLEc bind directly with NF-κβ factor, as well as the activation of NF-κB via DHX9 (Figure 8). The results obtained suggest that DLEc could modulate the inflammation process at three important levels of the molecular pathway, since different DLEc proteins interact with IKB1 and TNF-α and NF-κβ factors. In parallel, we searched the DLEc for bioactive peptides that have experimentally shown a biological effect *in vitro* or *in vivo*. We found peptides with immunomodulatory; anti-inflammatory; phagocytosis-inducing; antihypertensive; anticancer; antioxidant; modulators of various metabolic pathways; antimicrobial and antithrombotic activities (Table 2).

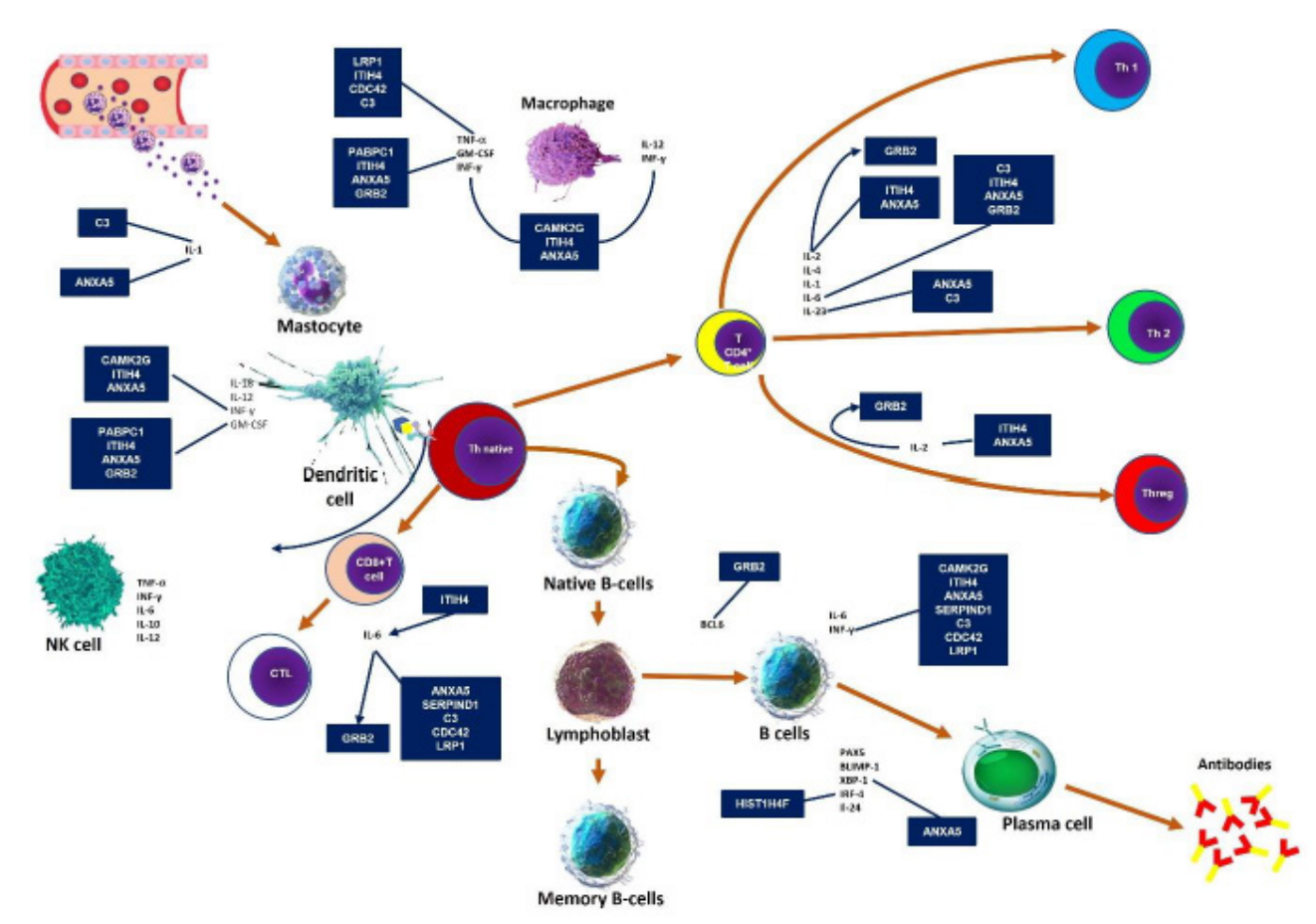

Figure 7: Immunomodulation of immune system cells by DLEc. The proteins signed in black induce maturation, differentiation, and activation of immune system cells; DLEc proteins signed in the navy-blue box may participate as regulators of these processes. The brown arrows indicate the normal physiological process, and the navy-blue arrows indicate direct interactions of the DLEc proteins with the proteins responsible for maturation, differentiation, and activation of immune system cells. arrows indicate activation and the lines only interaction (it is unknown whether they activate or inhibit their target protein).

Table 2: Bioactive peptides with known biological function present in DLEc proteins.

Bioactive Peptide	Biological Effect	DLEc Peptides	Repeats Number in DLEc	Reference
GLF	Immunomodulatory	• Ubiquitin-like modifier-activating enzyme 1 • Plastin-1, 2 and 3 • Myosin-9 • Talin-1 and 2 • Interleukin enhancer-binding factor 2	26	Kamau, et al. [24]
PAY	Immunomodulatory	• Annexin A5 • Complement C3 • Plexin domain-containing protein 2 • Piezo-type mechanosensitive ion channel component 1 • Transgelin-2	9	Ahn, et al. [25]

VH	Immunomodulatory	• Ubiquitin carboxyl-terminal hydrolase 7 • Complement C3 • Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) • Squamous Cell Carcinoma Antigen (SERPINB3) • Leukocyte elastase inhibitor (SERPINB1) Alpha-actinin-1	252	Lassoued, et al. [25]
LAN	Immunomodulatory	• Spectrin alpha chain, non-erythrocytic 1 • Myosin-9 • 6-phosphogluconate dehydrogenase, decarboxylating • Vinculin • Talin-1	28	Lassoued, et al. [25]
IRW	Immunomodulatory	• Aconitate hydratase, mitochondrial	1	Majumder, et al. [26]
IQW	Immunomodulatory	• Fibronectin • U6 snRNA-associated Sm-like protein LSm5 • Prolow-density lipoprotein receptor-related protein 1 • Transgelin-2 • Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	1	Majumder, et al. [26]
FELL	Immunomodulatory	• DNA damage-binding protein 1 • Aminopeptidase B • Acidic leucine-rich nuclear phosphoprotein 32 family member E	1	Lassoued, et al. [25]
LPHF	Immunomodulatory	• U5 small nuclear ribonucleoprotein 200 kDa helicase	1	Yoshikawa, et al. [37]
QRPR	Immunomodulatory	• Eukaryotic translation initiation factor 4H • Methanethiol oxidase	1	Chatterjee, et al. [38]
RQRK	Immunomodulatory	• U5 small nuclear ribonucleoprotein 200 kDa helicase • Peregrin • Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	1	Dia et al. [39]
VLER	Antiinflammatory	• Glutathione S-transferase Mu 2 • 4-trimethylaminobutyraldehyde dehydrogenase • Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	1	He at al. [40]
VLLF	Anti-inflammatory	• Spectrin beta chain, non-erythrocytic 1 • Heat shock protein HSP 90-beta • Basement membrane-specific heparan sulfate proteoglycan core protein • Heterogeneous nuclear ribonucleoprotein L	1	He at al. [40]
LFGK	Anti-inflammatory	• Spectrin beta chain, non-erythrocytic 1 Polyadenylate-binding protein 1 • Myosin light chain kinase, smooth muscle • Glutathione S-transferase A4 • UDP-glucose: glycoprotein glucosyltransferase 1	1	He, et al. [40]
LPF	Anti-inflammatory	• Ubiquitin-like modifier-activating enzyme 1 • Myosin-9 • Laminin subunit gamma 1 • Cytoplasmic aconitate hydratase • Phosphoglycerate mutase 2	19	Wang, et al. [41]

VPP	Anti-inflammatory and antihypertensive	• Complement C3 • Fibronectin • Filamin-A and B • Ras-related protein Rab-11A • Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	28	Bhat, et al. [7,12]
IPP	Anti-inflammatory and antihypertensive	• Polyubiquitin-C • Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) • Fibronectin • Complement C3 • Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	27	Bhat, et al. [42-43]
AFL	Antihypertensive	• Ras-related protein Rap-1ª • Myosin light chain kinase, smooth muscle • Piezo-type mechanosensitive ion channel component 1 • Ras-related protein Rab-5A • Serine/threonine-protein phosphatase 2A activator	37	Admassu, et al. [44]
FAL	Antihypertensive	• Leukocyte elastase inhibitor (SERPINB1) • U5 small nuclear ribonucleoprotein 200 kDa helicase • Laminin subunit beta 1 • Piezo-type mechanosensitive ion channel component 1 • Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	23	Admassu, et al. [44]
MKP	Antihypertensive	• Annexin A5 • Spectrin alpha chain, non-erythrocytic 1 • Aminopeptidase B • Xaa-Pro dipeptidase • Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	5	Majumder, et al. [36]
SVAR	Antihypertensive	• Keratin, type II cytoskeletal 1 • Plastin-1 • Glyoxalase domain-containing protein 4 • Prolow-density lipoprotein receptor-related protein 1 • Aldehyde dehydrogenase, mitochondrial	3	Lafarga, et al. 2016 [45]
LKP	Antihypertensive	• Heterogeneous nuclear ribonucleoprotein U-like protein • Septin-2 • Serpin B10 • Talin-2 • Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	40	Bhat, et al. [42]
LRP	Antihypertensive	• Complement C3 • Fibronectin • Piezo-type mechanosensitive ion channel component 1 • Ras-related C3 botulinum toxin substrate 2 • Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	26	Bhat et al. [42]

QPK	Anticancer	• Ubiquitin carboxyl-terminal hydrolase 7 • Elongation factor 1-gamma • Exportin-2 • Piezo-type mechanosensitive ion channel component 1 • Eukaryotic translation initiation factor 4 gamma	9	Huang et al. [27]
WTP	Anticancer	• Fibronectin • Transforming protein RhoA • Betaine--homocysteine S-methyltransferase 1 • Exportin-2 • Thioredoxin-dependent peroxide reductase, mitochondrial	1	Wang et al. [28]
WPP	Anticancer	• Vacuolar protein sorting-associated protein 13A • Putative tubulin-like protein alpha-4B • Heterogeneous nuclear ribonucleoprotein U-like protein • Vacuolar protein sorting-associated protein 13A	1	Chi et al. [29]
KLH	Anticancer	• Leukocyte elastase inhibitor (SERPINB1) • Vimentin • Talin-1 • DNA damage-binding protein 1 • Coronin-1C	16	Lamm et al. [46,12]
VPY	Antioxidant	• Ubiquitin-like modifier-activating enzyme 1 • Complement C3 • Basement membrane-specific heparan sulfate proteoglycan core protein • 26S proteasome non-ATPase regulatory subunit 2 • DNA excision repair protein ERCC-8	4	Kovacs et al. [28]
IQW	Antioxidant	• Fibronectin • U6 snRNA-associated Sm-like protein LSm5 • Prolow-density lipoprotein receptor-related protein 1 • Transgelin-2 • Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	1	Majumder et al. [36]
SHR	Antioxidant	• U5 small nuclear ribonucleoprotein 200 kDa helicase • Aldehyde dehydrogenase, mitochondrial • Aminopeptidase N • Basement membrane-specific heparan sulfate proteoglycan core protein • Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	12	Manso et al. [47]
DLEE	Antioxidant	• Myosin-9 • Myosin-10 • F-actin-capping protein subunit alpha-2 • 2-oxoglutarate dehydrogenase-like, mitochondrial • Ras-related protein Ral-B	7	Xing et al. [48]

DPR	Hypocholesterolemia	• Rab GDP dissociation inhibitor beta U6 snRNA-associated Sm-like protein LSm5 • Coronin-1C • F-actin-capping protein subunit alpha-1 • Endoplasmin • F-actin-capping protein subunit alpha-2	21	Takenaka et al. [49]
SY	Triglyceride synthesis inhibition	• Ubiquitin carboxyl-terminal hydrolase 7 • Complement C3 • Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) • Squamous Cell Carcinoma Antigen, SCCA1 (SERPINB3) • Leukocyte elastase inhibitor (SERPINB1)	350	Inoue et al. [50]
VK	Triglyceride synthesis inhibition	• Polyubiquitin-C • Ubiquitin carboxyl-terminal hydrolase 7 • Ubiquitin recognition factor in ER-associated degradation protein 1 • Ubiquitin-like modifier-activating enzyme 1 • Complement C3 • Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) • Leukocyte elastase inhibitor (SERPINB1) • Annexin A5	700	Inoue et al. [50]
KA	Triglyceride synthesis inhibition	• Polyubiquitin-C • Ubiquitin carboxyl-terminal hydrolase 7 • Ubiquitin recognition factor in ER-associated degradation protein 1 • Ubiquitin-like modifier-activating enzyme 1 • Complement C3 • Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) • Squamous Cell Carcinoma Antigen, SCCA1 (SERPINB3) • Leukocyte elastase inhibitor (SERPINB1) • Annexin A5	900	Inoue et al. [50]
WV	Hypoglycemic	• Complement C3 • Squamous Cell Carcinoma Antigen, SCCA1 (SERPINB3) • Leukocyte elastase inhibitor (SERPINB1) • Aminopeptidase N • Aconitate hydratase, mitochondrial	124	Arrutia et al. [51]
LI	Hypoglycemic	• Polyubiquitin-C • Ubiquitin carboxyl-terminal hydrolase 7 • Ubiquitin-like modifier-activating enzyme 1 • Complement C3 • Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) • Squamous Cell Carcinoma Antigen, SCCA1 (SERPINB3) • Annexin A5	800	Morato et al. [11]
KRDS	Antithrombotic	• Eukaryotic translation initiation factor 4 gamma 3	1	Drouet et al. [52]
WGC	Antithrombotic	• Alpha-actinin-1 • Prelamin-A/C	1	Ku et al. [13]

SQL	Antithrombotic	• Keratin, type I cytoskeletal 9 and 10 • Spectrin alpha chain, non-erythrocytic 1 • Myosin-9 • Vinculin	67	Su et al. [53]
FHG	Antithrombotic	• Plastin-2 • Tyrosine-protein kinase CSK • Heterogeneous nuclear ribonucleoprotein H3 • Glutamate dehydrogenase 1, mitochondrial • Heparin cofactor 2	3	Jang et al. [54]

Discussion

The 2D proteomic characterization and the study of structure-function relationship of peptides contained in DLEc showed at least eighty major spots in molecular weight ranges between 38 and 6 kDa. The first interpretation of this result refutes at a stroke the long-standing assertion that transfer factor is a mixture of a small number of short amino acid sequences smaller than 5 kDa (Kirkpatrick, et al. [30]). Each spot corresponds to a protein or peptide with a characteristic molecular weight and isoelectric point, which indicated that the number of peptides far exceeded the hypothesis that remained valid for decades. For the sequencing analysis by mass spectrometry, we selected only the most abundant spots. Results showed the presence of structural proteins; enzymes ribonucleoproteins; constituent peptides of energy metabolism enzymes; heat shock proteins; interestingly, several ubiquitin constituent peptides, and various unidentified proteins. Recently, Vallejo-Castillo et al 2022, reported the proteome of DLE from human blood peripheral leucocytes, showing the presence of several peptides of at least from twenty-two human proteins, including actin, ankyrin-1, hemoglobin b-subunit, C3 complement protein, a-synuclein and as principal component, polyubiquitin-C that have immune regulatory effects. We found here that DLEc contains actins 1, 2, 4; C3 complement protein, and similar to previous work, ubiquitin protein peptides as well as some others related to the protein degradation process, such as polyubiquitin-C, ubiquitin carboxyl-terminal hydrolase 7, ubiquitin fusion degradation protein 1 homolog, ubiquitin-like modifier-activating enzyme 1, proteasome subunit alpha type-1, 2, 5, 7, proteasome subunit beta type-1, 2, 3, 26S proteasome non-ATPase regulatory subunit 2, and SUMO-activating enzyme subunit 1.

Taking into consideration that due the diversity, number, and abundance of peptides obtained by two-dimensional proteomic analysis exceeds eighty peptides, characterizing the DLEc proteome by this route will produce the loss of those peptides poorly represented, which could have relevant immune modulatory effects. Therefore, the analysis was performed using nanoLC-MS/MS orbitrap. After analyzed the entire proteome by bioinformatic analysis, about 302 peptides were identified, from those mostly represented and others with minimal abundance. Human DLE peptides are derived from 539 proteins grouped into eight clusters: I. Ankyrin-1, II. α-subunit of hemoglobin, III. Subunit β of hemoglobin, IV. Calpastatin, V. α-synuclein, VI. Cytoplasmic actin, VII. Polyubiquitin C and VIII. thymosin, as reported by Vallejo-Castillo et al. 2022. Although the polyubiquitin C cluster is one of the least abundant, they found by mass spectrometry that this protein contributes a greater number of peptides to the total DLE content. This work group highlighted the importance of this monomeric protein in a murine model (BAL/C mice) with Herpes Simplex Virus type 1 infection. Administration of DLE enriched with the monomeric ubiquitin increased the survival of mice compared to unenriched DLE, and even survival decreased further when ubiquitin is depleted from DLE. The authors continue that monomeric ubiquitin is the major bioactive component of the complex mixture of human DLE.

In our study we found that DLEc is an even more complex mixture than human DEL because it is derived from lymphoid tissue, which is rich in multiple cell subpopulations, compared to human DLE which is derived solely from blood leukocytes. Peptides correspond to a wide variety of proteins with structural, metabolic, and regulatory functions in various cellular processes. Interestingly, in DLEc we identified peptides possessed by human DLE, such as polyubiquitin C, complement protein C3 and cytoplasmic actin. Its knowledge lacks practical and medical importance if we are not able to define and associate the molecular pathways in which it participates, with special emphasis on those pathways that some groups in the world have experimentally demonstrated that different DLE are able to modulate physiological functions and favor the adjuvant or resolution of specific disorders. Immunomodulatory molecules can stimulate or suppress the immune response acting at various levels to selectively inhibit or activate populations or subpopulations of immune cells, such as lymphocytes, macrophages, neutrophils, NK killer cells and cytotoxic cells; or to modulate the production of soluble mediators such as cytokines. Among the immunomodulatory molecules are cytokines such as IL-1, INF g, IL-2, IL-5, IL-12, TNFa, IL-18 and GM-CSF, which can act directly or in-

directly, being able to modulate the humoral and cellular immune response. Immunomodulators are frequently used as therapy for immunodeficiencies, cancer, hepatitis and in hematopoietic recovery (Nicholas, et al. [20]).

Interestingly, in DLEc we found peptides derived from five proteins that can potentially interact with human proteins involved in the immune system response. Annexin A5, SERPIND1, C3, SERPINB3 and ITIH4 can interact with different proteins related to the modulation of pro- and anti-inflammatory interleukins; activation of immune cells; chemotaxis, as well as activation of the complement system. We also identified that annexin 5, ITIH4, C3, CDC42, LRP1, CALM3, YWHAB, DHX9, ILF2, YWHAE, ILF2, and RAN can interact with TNFR, IKB1, NF-κB factor. Nuclear factor enhancer of activated B-cell kappa light chains (NF-κβ) is a protein complex that controls DNA transcription. This protein plays a key role in the regulation of the immune response elicited by different stimulus. Defective regulation of NF-kB is associated with cancer; inflammatory and autoimmune diseases; septic shock; viral infections or inappropriate immune development (Echeverri et al. [31]). NF-κβ can be activated by toxins, bacteria, viruses, parasites, or oxidative stress, however, the main molecular pathway for its activation is through the TNFR receptor, which can be activated by different cytokines or by tumor necrosis factor (TNF). In turn, when activated, it passes the signal downstream activating the adaptor proteins TRAF2-IKK, this allows inducing the phosphorylation of IKB1 (under normal conditions it is heterodimerized with NF-κβ) and, therefore, the release and activation of NF-κβ (Christian, et al. [32,33]). Once NF-κB is activated, it is translocated to the nucleus and transcribes various genes that protect and induce proliferation of cells that should otherwise die by apoptosis to prevent damage from spreading. In addition, it also regulates the transcription of genes involved in different cellular processes such as the maturation of different immune cells, the regulation of the cell cycle, the biosynthesis of inflammatory and proinflammatory cytokines, among others (Chen, et al. [34]). Our previous studies did in inflammatory diseases (Acosta et al. [16-18]) suggest that DLEc treatment prevent uncoupling of IκB from NFkB and, therefore, preventing it from translocating to the nucleus to carry out transcription of proinflammatory genes.

Using the proteome obtained with nanoLC-MS/MS, we perform interactomes to identify putative molecular pathways modulated by the DLEc peptides. The systematic study and comparison of the proteome in different metabolic or pathological situations allows us to identify those proteins whose presence, absence or alteration correlates with certain physiological stages (Aslam, et al. [33-59]). Taking up diverse research from different groups in the world and our own research carried out with DLEc, we focus the OMIC analysis on the capacity of DLEc to modulate different molecular pathways in the cell, with particular emphasis on those with experimental evidence and related to the immunomodulatory processes that are deregulated in the health-disease binomial. Alternatively, we searched for peptides with proven biological effect *in vitro* or *in vivo* and found that immune and ubiquitin related proteins are rich in bioactive peptides with immunomodulatory, anti-inflammatory, antihypertensive, anticancer, antioxidant, hypocholesterolemic, triglyceride synthesis inhibitor, hypoglycemic, antithrombotic, and antimicrobial functions (Table 2). Thus, *in silico* analyses together presented in this work strongly suggest the existence of several bioactive peptides with enormous potential as pharmacological adjuvant in different pathologies. Undoubtedly, once the proteome of DLE has been determined, future research should focus on determining its mechanisms of action, bioavailability, and pharmacological kinetics to be considered in a new, more natural, less toxic, self-sufficient, and human-friendly therapeutic that can help in the control of a wide variety of pathological conditions.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Supplementary Table 1. PSMs, total number of identified peptide sequences; AAs, amino acids; MW (kDa), molecular weight in kilodaltons and pI, Isoelectric point.

	Accession	Coverage	# PSMs	# Peptides	# AAs	MW (kDa)	pI	Score	DLEc proteins
1	P13796	29.82	307	16	627	70.2	5.43	7798.98	Plastin-2
2	P13797	16.35	226	10	630	70.8	5.6	5805.15	Plastin-3
3	P12814	54.93	168	38	892	103	5.41	5438.04	Alpha-actinin-1
4	O43707	40.72	131	32	911	104.8	5.44	4293.83	Alpha-actinin-4
5	P06733	27.65	75	7	434	47.1	7.39	2696.91	Alpha-enolase
6	P09104	13.82	75	4	434	47.2	5.03	2607.52	Gamma-enolase
7	P35609	17.9	79	16	894	103.8	5.45	2518.32	Alpha-actinin-2

8	P60709	67.47	103	16	375	41.7	5.48	2499.86	Actin, cytoplasmic 1
9	P13929	17.28	61	5	434	47	7.71	2244.77	Beta-enolase
10	P13645	39.9	44	15	584	58.8	5.21	1669.8	Keratin, type I cytoskeletal 10
11	Q13813	16.34	54	29	2472	284.4	5.35	1649.08	Spectrin alpha chain, brain
12	P11021	30.28	47	16	654	72.3	5.16	1531.75	78 kDa glucose-regulated protein
13	P04264	29.97	49	12	644	66	8.12	1528.16	Keratin, type II cytoskeletal 1
14	P11142	37.31	46	18	646	70.9	5.52	1456.63	Heat shock cognate 71 kDa protein
15	P62258	48.63	45	13	255	29.2	4.74	1355.24	14-3-3 protein epsilon
16	P55072	25.31	39	14	806	89.3	5.26	1282.61	Transitional endoplasmic reticulum ATPase
17	P50395	18.65	27	6	445	50.6	6.47	1082.94	Rab GDP dissociation inhibitor beta
18	P54652	16.74	29	10	639	70	5.74	1034.49	Heat shock-related 70 kDa protein 2
19	P06753	29.23	37	10	284	32.8	4.72	1034.49	Tropomyosin alpha-3 chain
20	P08758	10.63	26	3	320	35.9	5.05	994.34	Annexin A5
21	P68032	28.91	32	9	377	42	5.39	976.65	Actin, alpha cardiac muscle 1
22	P35579	8.83	24	13	1960	226.4	5.6	965.26	Myosin-9
23	P63104	35.51	33	8	245	27.7	4.79	940	14-3-3 protein zeta/delta
24	P09493	16.9	37	7	284	32.7	4.74	937.16	Tropomyosin alpha-1 chain
25	P52209	18.01	23	6	483	53.1	7.23	909.37	6-phosphogluconate dehydrogenase, decarboxylating
26	P60174	24.13	26	7	286	30.8	5.92	900.33	Triosephosphate isomerase
27	P06899	26.19	17	3	126	13.9	10.32	845.8	Histone H2B type 1-J
28	P61224	41.3	26	7	184	20.8	5.78	820.11	Ras-related protein Rap-1b
29	P62158	54.36	32	7	149	16.8	4.22	773.39	Calmodulin
30	P35527	32.42	21	11	623	62	5.24	758.42	Keratin, type I cytoskeletal 9
31	P62834	41.3	23	6	184	21	6.67	745.61	Ras-related protein Rap-1A
32	Q71DI3	23.53	16	1	136	15.4	11.27	735.27	Histone H3.2
33	P31943	15.81	22	5	449	49.2	6.3	724.59	Heterogeneous nuclear ribonucleoprotein H
34	P18206	18.17	29	14	1134	123.7	5.66	722.95	Vinculin
35	P27348	29.39	25	6	245	27.7	4.78	722.92	14-3-3 protein theta
36	P61981	20.65	22	4	247	28.3	4.89	709.79	14-3-3 protein gamma
37	P08670	11.37	23	6	466	53.6	5.12	697.88	Vimentin
38	P55786	17.74	22	13	919	103.2	5.72	661.63	Puromycin-sensitive aminopeptidase
39	O75369	5.84	26	11	2602	278	5.73	657.63	Filamin-B
40	Q01105	33.79	19	6	290	33.5	4.32	627.51	Protein SET
41	P61978	15.12	14	5	463	50.9	5.54	604.7	Heterogeneous nuclear ribonucleoprotein K
42	Q99598	6.55	15	1	290	33.1	6.55	599.65	Translin-associated protein X
43	P47756	31.05	17	7	277	31.3	5.59	586.29	F-actin-capping protein subunit beta
44	P07900	9.15	18	5	732	84.6	5.02	575.12	Heat shock protein HSP 90-alpha
45	Q14651	13.51	22	8	629	70.2	5.41	561.19	Plastin-1
46	Q01082	4.95	16	8	2364	274.4	5.57	538.29	Spectrin beta chain, brain 1
47	P35749	4.01	14	6	1972	227.2	5.5	511.34	Myosin-11
48	P35908	18.78	17	10	639	65.4	8	510.14	Keratin, type II cytoskeletal 2 epidermal
49	Q16777	49.61	13	4	129	14	10.9	508.4	Histone H2A type 2-C
50	P39687	16.87	12	3	249	28.6	4.09	505.04	Acidic leucine-rich nuclear phosphoprotein 32 family member A
51	P10809	13.44	14	5	573	61	5.87	496.12	60 kDa heat shock protein, mitochondrial

52	P62805	41.75	18	5	103	11.4	11.36	490.14	Histone H4
53	P31946	21.14	18	4	246	28.1	4.83	479.26	14-3-3 protein beta/alpha
54	A6NL28	12.11	15	3	223	26.3	4.51	476.68	Putative tropomyosin alpha-3 chain-like protein
55	Q15631	17.11	10	2	228	26.2	6.44	438.66	Translin
56	P30101	9.31	15	4	505	56.7	6.35	421.64	Protein disulfide-isomerase A3
57	P11047	5.97	14	9	1609	177.5	5.12	404.5	Laminin subunit gamma-1
58	P14618	14.5	14	6	531	57.9	7.84	391.28	Pyruvate kinase isozymes M1/M2
59	P21333	3.29	12	5	2647	280.6	6.06	386.38	Filamin-A
60	Q04917	17.89	16	4	246	28.2	4.84	384.83	14-3-3 protein eta
61	Q14195	11.58	12	4	570	61.9	6.49	380.25	Dihydropyrimidinase-related protein 3
62	Q9Y490	3.86	11	8	2541	269.6	6.07	379.52	Talin-1
63	Q14315	4.73	14	8	2725	290.8	5.97	379.08	Filamin-C
64	P11940	10.06	9	5	636	70.6	9.5	375.38	Polyadenylate-binding protein 1
65	Q12905	27.44	14	7	390	43	5.26	356.63	Interleukin enhancer-binding factor 2
66	P07237	11.22	14	6	508	57.1	4.87	351.82	Protein disulfide-isomerase
67	Q96KK5	50	10	4	128	13.9	10.89	333.81	Histone H2A type 1-H
68	P35580	4.05	10	6	1976	228.9	5.54	333.08	Myosin-10
69	Q01995	14.93	11	3	201	22.6	8.84	329.65	Transgelin
70	P40925	19.46	11	5	334	36.4	7.36	316.01	Malate dehydrogenase, cytoplasmic
71	P46926	19.72	11	4	289	32.6	6.92	310.59	Glucosamine-6-phosphate isomerase 1
72	P14136	4.4	11	2	432	49.8	5.52	306.46	Glial fibrillary acidic protein
73	P12277	7.87	6	2	381	42.6	5.59	289.09	Creatine kinase B-type
74	P06748	7.14	6	1	294	32.6	4.78	257.78	Nucleophosmin
75	O75368	35.96	9	4	114	12.8	5.25	251.79	SH3 domain-binding glutamic acid-rich-like protein
76	P62314	16.81	8	1	119	13.3	11.56	244.1	Small nuclear ribonucleoprotein Sm D1
77	P27797	13.43	11	4	417	48.1	4.44	236.4	Calreticulin
78	P22626	4.53	5	1	353	37.4	8.95	232.99	Heterogeneous nuclear ribonucleoproteins A2/B1
79	P08238	6.77	7	4	724	83.2	5.03	231.93	Heat shock protein HSP 90-beta
80	P36873	8.05	5	2	323	37	6.54	230.99	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit
81	Q16531	4.56	9	4	1140	126.9	5.26	230.63	DNA damage-binding protein 1
82	P49419	4.45	5	2	539	58.5	7.99	230.56	Alpha-aminoadipic semialdehyde dehydrogenase
83	Q1KMD3	8.03	7	5	747	85.1	4.91	229.75	Heterogeneous nuclear ribonucleoprotein U-like protein 2
84	P37837	3.26	8	1	337	37.5	6.81	222.67	Transaldolase OS=Homo sapiens GN=TALDO1 PE=1 SV=2 - [TALDO_HUMAN]
85	P60953	20.94	9	3	191	21.2	6.55	218.11	Cell division control protein 42 homolog
86	P23526	10.88	7	5	432	47.7	6.34	215.22	Adenosylhomocysteinase
87	P04259	6.56	6	3	564	60	8	211.48	Keratin, type II cytoskeletal 6B
88	Q14974	3.2	8	2	876	97.1	4.78	208.57	Importin subunit beta-1
89	Q9ULV4	16.46	10	5	474	53.2	7.08	208.11	Coronin-1C
90	P35080	10	3	1	140	15	6.99	206.59	Profilin-2
91	P00338	9.64	8	3	332	36.7	8.27	205.61	L-lactate dehydrogenase A chain

92	Q13557	8.02	7	3	499	56.3	7.25	203.77	Calcium/calmodulin-dependent protein kinase type II subunit delta
93	P21399	2.81	6	2	889	98.3	6.68	200.82	Cytoplasmic aconitate hydratase
94	P06744	5.38	6	2	558	63.1	8.32	197.29	Glucose-6-phosphate isomerase
95	P68402	3.93	7	1	229	25.6	5.92	193.88	Platelet-activating factor acetylhydrolase IB subunit beta
96	P02545	5.12	7	3	664	74.1	7.02	193.57	Lamin-A/C
97	P08133	2.82	8	2	673	75.8	5.6	192.88	Annexin A6
98	P24844	24.42	7	4	172	19.8	4.92	189.5	Myosin regulatory light polypeptide 9
99	Q9NQW7	4.82	6	2	623	69.9	5.67	189.19	Xaa-Pro aminopeptidase 1
100	P15259	16.21	7	2	253	28.7	8.88	188.98	Phosphoglycerate mutase 2
101	P84243	23.53	5	1	136	15.3	11.27	186.84	Histone H3.3
102	P62316	31.36	4	3	118	13.5	9.91	182.29	Small nuclear ribonucleoprotein Sm D2
103	P52907	15.73	8	3	286	32.9	5.69	181.93	F-actin-capping protein subunit alpha-1
104	P00558	4.08	5	1	417	44.6	8.1	176.34	Phosphoglycerate kinase 1
105	P18669	9.84	6	2	254	28.8	7.18	175.71	Phosphoglycerate mutase 1
106	P61604	13.73	4	1	102	10.9	8.92	175.32	10 kDa heat shock protein, mitochondrial
107	P0C0S5	35.16	6	3	128	13.5	10.58	174.81	Histone H2A.Z
108	Q92841	3.16	5	2	729	80.2	8.27	170.37	Probable ATP-dependent RNA helicase DDX17
109	P07858	5.9	2	1	339	37.8	6.3	168.73	Cathepsin B
110	P19105	18.13	6	3	171	19.8	4.81	167.28	Myosin regulatory light chain 12A
111	P07195	6.29	6	2	334	36.6	6.05	165.86	L-lactate dehydrogenase B chain
112	P14625	5.11	4	3	803	92.4	4.84	165.56	Endoplasmin
113	Q06830	9.05	5	2	199	22.1	8.13	164.55	Peroxiredoxin-1
114	P05388	6.94	4	2	317	34.3	5.97	162.21	60S acidic ribosomal protein P0
115	Q9H4A4	4.15	6	2	650	72.5	5.74	160.07	Aminopeptidase B
116	P35221	3.2	4	1	906	100	6.29	158.78	Catenin alpha-1
117	P47755	15.73	6	3	286	32.9	5.85	155.8	F-actin-capping protein subunit alpha-2
118	P41240	5.11	7	2	450	50.7	7.06	153.49	Tyrosine-protein kinase CSK
119	P62318	23.81	6	2	126	13.9	10.32	152.8	Small nuclear ribonucleoprotein Sm D3
120	P61026	16.5	7	3	200	22.5	8.38	152.65	Ras-related protein Rab-10
121	P61970	11.02	3	1	127	14.5	5.38	151.98	Nuclear transport factor 2
122	P31942	4.91	5	1	346	36.9	6.87	150.7	Heterogeneous nuclear ribonucleoprotein H3
123	Q13555	9.5	5	3	558	62.6	7.83	148.91	Calcium/calmodulin-dependent protein kinase type II subunit gamma
124	O75643	0.7	4	1	2136	244.4	6.06	147.96	U5 small nuclear ribonucleoprotein 200 kDa helicase
125	P0CG48	44.67	6	3	685	77	7.66	145.08	Polyubiquitin-C
126	P61586	19.69	5	2	193	21.8	6.1	143.04	Transforming protein RhoA
127	P00367	4.84	6	2	558	61.4	7.8	141.31	Glutamate dehydrogenase 1, mitochondrial
128	Q9GZS3	6.56	3	1	305	33.6	5.47	140.34	WD repeat-containing protein 61
129	Q15185	15.63	7	2	160	18.7	4.54	138.15	Prostaglandin E synthase 3
130	Q9HAV0	8.53	3	2	340	37.5	6	137.33	Guanine nucleotide-binding protein subunit beta-4
131	Q13554	3.75	4	1	666	72.6	7.27	136.71	Calcium/calmodulin-dependent protein kinase type II subunit beta
132	P07942	2.07	5	3	1786	197.9	4.94	136.34	Laminin subunit beta-1 O

133	P14678	3.33	3	1	240	24.6	11.19	133.11	Small nuclear ribonucleoprotein-associated proteins B and B'
134	P28330	2.79	4	1	430	47.6	7.8	131.19	Long-chain specific acyl-CoA dehydrogenase, mitochondrial
135	Q13162	10.7	5	3	271	30.5	6.29	130.69	Peroxiredoxin-4
136	Q9HC38	4.15	3	1	313	34.8	5.6	130.1	Glyoxalase domain-containing protein 4
137	O43390	6.64	6	4	633	70.9	8.13	126.79	Heterogeneous nuclear ribonucleoprotein R
138	P26641	5.03	5	1	437	50.1	6.67	124.37	Elongation factor 1-gamma
139	Q92928	22.89	6	4	201	22	5.43	121.14	Putative Ras-related protein Rab-1C
140	P02751	1.93	5	2	2386	262.5	5.71	120.35	Fibronectin
141	Q9UHX1	1.97	3	2	559	59.8	5.29	120.1	Poly(U)-binding-splicing factor PUF60
142	Q93009	3.9	3	3	1102	128.2	5.55	119.75	Ubiquitin carboxyl-terminal hydrolase 7
143	Q9Y333	20	1	1	95	10.8	6.52	116.72	U6 snRNA-associated Sm-like protein LSm2
144	Q15084	2.95	2	1	440	48.1	5.08	116.27	Protein disulfide-isomerase A6
145	P30837	2.9	3	1	517	57.2	6.8	113.43	Aldehyde dehydrogenase X, mitochondrial
146	P00390	4.79	4	2	522	56.2	8.5	107.5	Glutathione reductase, mitochondrial
147	P12429	4.64	2	1	323	36.4	5.92	105.78	Annexin A3
148	Q58FF6	4.16	3	2	505	58.2	4.73	101.88	Putative heat shock protein HSP 90-beta 4
149	P28161	4.13	3	1	218	25.7	6.37	100.65	Glutathione S-transferase Mu 2
150	Q96E39	6.92	3	2	390	42.1	9.89	100.22	RNA binding motif protein, X-linked-like-1
151	P35030	4.28	2	1	304	32.5	7.49	100	Trypsin-3
152	P60660	5.3	3	1	151	16.9	4.65	98.33	Myosin light polypeptide 6
153	O00629	3.26	2	1	521	57.9	4.96	98	Importin subunit alpha-4
154	P62491	6.02	2	1	216	24.4	6.57	96.24	Ras-related protein Rab-11A
155	P09972	3.85	2	1	364	39.4	6.87	95.52	Fructose-bisphosphate aldolase C
156	Q9Y4Y9	36.26	3	2	91	9.9	4.54	95.15	U6 snRNA-associated Sm-like protein LSm5
157	P46940	1.15	3	2	1657	189.1	6.48	92.97	Ras GTPase-activating-like protein IQGAP1
158	Q15056	5.24	2	1	248	27.4	7.23	89.4	Eukaryotic translation initiation factor 4H
159	P98160	0.73	3	3	4391	468.5	6.51	88.81	Basement membrane-specific heparan sulfate proteoglycan core protein
160	P11310	2.14	2	1	421	46.6	8.37	88.33	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial
161	Q9NVA2	7.69	4	2	429	49.4	6.81	88.3	Septin-11
162	Q13228	3.18	2	1	472	52.4	6.37	87.02	Selenium-binding protein 1
163	P15586	4.71	3	2	552	62	8.31	86.42	N-acetylglucosamine-6-sulfatase
164	P02533	5.08	2	2	472	51.5	5.16	83.7	Keratin, type I cytoskeletal 14
165	P26368	5.05	4	2	475	53.5	9.09	83.34	Splicing factor U2AF 65 kDa subunit
166	P50452	2.67	3	1	374	42.7	5.57	82.64	Serpin B8
167	Q6QEF8	2.75	2	1	472	52.7	5.96	82.57	Coronin-6
168	P28066	10.37	3	2	241	26.4	4.79	82.41	Proteasome subunit alpha type-5
169	P51149	13.04	3	2	207	23.5	6.7	78.6	Ras-related protein Rab-7a
170	O43396	2.42	3	1	289	32.2	4.96	77.96	Thioredoxin-like protein 1
171	Q9BTE7	3.8	2	1	237	27.5	5.58	77.32	DCN1-like protein 5

172	Q92890	8.14	3	2	307	34.5	6.7	76.25	Ubiquitin fusion degradation protein 1 homolog
173	P04844	2.22	2	1	631	69.2	5.69	76.19	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2
174	Q9Y294	7.35	1	1	204	23	4.41	75.73	Histone chaperone ASF1A
175	P13861	4.21	1	1	404	45.5	5.07	75.56	cAMP-dependent protein kinase type II-alpha regulatory subunit
176	P15121	3.16	3	1	316	35.8	6.98	75.31	Aldose reductase
177	P01111	6.35	2	1	189	21.2	5.17	75.04	GTPase NRas
178	Q03013	4.13	3	1	218	25.5	5.9	74.91	Glutathione S-transferase Mu 4
179	Q93088	6.4	2	2	406	45	7.03	74.65	Betaine--homocysteine S-methyltransferase 1
180	P38646	2.36	1	1	679	73.6	6.16	74.08	Stress-70 protein, mitochondrial
181	Q01518	3.79	3	2	475	51.9	8.06	71.54	Adenylyl cyclase-associated protein 1
182	Q9BTT0	4.48	3	1	268	30.7	3.85	71.17	Acidic leucine-rich nuclear phosphoprotein 32 family member E
183	P25787	8.12	2	1	234	25.9	7.43	70.24	Proteasome subunit alpha type-2
184	P05452	5.94	3	1	202	22.5	5.67	67.55	Tetranectin
185	P05546	2.2	2	1	499	57	6.9	67.29	Heparin cofactor 2
186	P62993	11.06	3	2	217	25.2	6.32	67.1	Growth factor receptor-bound protein 2
187	Q9Y371	3.29	2	1	365	40.8	6.04	66.62	Endophilin-B1
188	P09601	4.17	2	1	288	32.8	8.25	66.09	Heme oxygenase 1
189	P15144	1.34	1	1	967	109.5	5.48	64.26	Aminopeptidase N
190	P49189	4.45	2	2	494	53.8	5.87	64.06	4-trimethylaminobutyraldehyde dehydrogenase
191	P17661	4.04	3	2	470	53.5	5.27	63.55	Desmin
192	O00148	2.34	1	1	427	49.1	5.68	61.08	ATP-dependent RNA helicase DDX39
193	Q9H853	5.81	2	1	241	27.5	7.83	61.05	Putative tubulin-like protein alpha-4B
194	A8MWD9	15.79	1	1	76	8.5	8.84	60.94	Small nuclear ribonucleoprotein G-like protein
195	P01024	1.14	2	2	1663	187	6.4	59.16	Complement C3
196	P25786	5.7	1	1	263	29.5	6.61	59.11	Proteasome subunit alpha type-1
197	Q99798	2.05	1	1	780	85.4	7.61	59.03	Aconitate hydratase, mitochondrial
198	O43447	7.91	2	1	177	19.2	8.07	58.96	Peptidyl-prolyl cis-trans isomerase H
199	P20618	5.81	1	1	241	26.5	8.13	58.63	Proteasome subunit beta type-1
200	P14866	4.92	1	1	589	64.1	8.22	58.24	Heterogeneous nuclear ribonucleoprotein L
201	A6NMY6	4.72	2	1	339	38.6	6.95	57.76	Putative annexin A2-like protein
202	P30050	15.15	3	2	165	17.8	9.42	56.9	60S ribosomal protein L12
203	P04406	4.18	1	1	335	36	8.46	56.83	Glyceraldehyde-3-phosphate dehydrogenase
204	P63208	11.66	2	1	163	18.6	4.54	55.55	S-phase kinase-associated protein 1
205	Q2TAY7	3.31	1	1	513	57.5	7.18	55.02	WD40 repeat-containing protein SMU1
206	Q15746	0.57	1	1	1914	210.6	6.15	54.54	Myosin light chain kinase, smooth muscle
207	P20933	3.47	2	1	346	37.2	6.28	53.61	N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase
208	P60981	16.97	2	2	165	18.5	7.85	53.6	Destrin
209	Q14247	2.18	2	1	550	61.5	5.4	53.57	Src substrate cortactin
210	P53999	8.66	2	1	127	14.4	9.6	53.54	Activated RNA polymerase II transcriptional coactivator p15
211	P55060	0.82	2	1	971	110.3	5.77	53.25	Exportin-2

212	P22314	1.32	1	1	1058	117.8	5.76	53.23	Ubiquitin-like modifier-activating enzyme 1
213	Q96BW5	3.15	1	1	349	39	6.52	52.99	Phosphotriesterase-related protein
214	O43143	1.51	1	1	795	90.9	7.46	51.96	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15
215	Q13200	2.09	2	1	908	100.1	5.2	51.47	26S proteasome non-ATPase regulatory subunit 2
216	P62304	11.96	2	1	92	10.8	9.44	51.46	Small nuclear ribonucleoprotein E
217	O60361	13.87	2	1	137	15.5	8.57	50.72	Putative nucleoside diphosphate kinase
218	Q14165	4.11	2	1	292	32.2	5.41	50.6	Malectin
219	P11234	3.4	2	1	206	23.4	6.62	50.42	Ras-related protein Ral-B
220	Q96M42	4.93	2	1	142	15.2	8	50.18	Uncharacterized protein C21orf129
221	P08243	1.6	3	1	561	64.3	6.86	50.14	Asparagine synthetase [glutamine-hydrolyzing]
222	Q6UX71	2.27	1	1	529	59.5	6.46	49.67	Plexin domain-containing protein 2
223	Q07954	0.24	2	1	4544	504.3	5.39	49.49	Prolow-density lipoprotein receptor-related protein 1
224	P29508	1.79	1	1	390	44.5	6.81	49.14	Serpin B3
225	Q9UBX5	2.46	2	1	448	50.1	4.73	48.98	Fibulin-5
226	P56537	9.8	1	1	245	26.6	4.68	48.75	Eukaryotic translation initiation factor 6
227	P04083	4.62	1	1	346	38.7	7.02	48.56	Annexin A1]
228	P31153	3.8	1	1	395	43.6	6.48	48.44	S-adenosylmethionine synthetase isoform type-2
229	Q9ULD0	1.78	2	2	1010	114.4	6.65	48.41	2-oxoglutarate dehydrogenase-like, mitochondrial
230	Q9BRF8	3.5	1	1	314	35.5	6.2	47.81	Calcineurin-like phosphoesterase domain-containing protein 1
231	O95777	16.67	1	1	96	10.4	4.48	47.77	U6 snRNA-associated Sm-like protein LSm8
232	Q10567	2.42	1	1	949	104.6	5.06	47.69	AP-1 complex subunit beta-1
233	Q92508	0.48	2	1	2521	286.6	7.47	47.09	Piezo-type mechanosensitive ion channel component 1
234	Q6IBS0	4.58	1	1	349	39.5	6.84	46.81	Twinfilin-2
235	Q9BUJ2	1.52	1	1	856	95.7	6.92	46.58	Heterogeneous nuclear ribonucleoprotein U-like protein 1
236	P84085	11.67	2	2	180	20.5	6.79	45.41	ADP-ribosylation factor 5
237	P20700	2.05	1	1	586	66.4	5.16	45.06	Lamin-B1
238	P62714	3.56	1	1	309	35.6	5.43	44.74	Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform
239	Q8TAT6	0.99	2	1	608	68.1	6.38	44.7	Nuclear protein localization protein 4 homolog
240	Q9C0K3	5.24	1	1	210	23.7	5.58	44.57	Actin-related protein 11
241	Q15019	4.43	1	1	361	41.5	6.6	44.4	Septin-2
242	P20339	5.12	1	1	215	23.6	8.15	44.25	Ras-related protein Rab-5A
243	P49720	12.2	2	2	205	22.9	6.55	44.06	Proteasome subunit beta type-3
244	Q8N6L0	1.6	1	1	562	62.7	4.68	43.9	Coiled-coil domain-containing protein 155
245	Q15257	3.63	1	1	358	40.6	5.94	42.44	Serine/threonine-protein phosphatase 2A regulatory subunit B' OS=Homo sapiens GN=PPP2R4 PE=1 SV=3 - [PTPA_HUMAN]
246	P49721	7.46	1	1	201	22.8	7.02	42.05	Proteasome subunit beta type-2
247	P48595	2.52	1	1	397	45.4	6.16	41.55	Serpin B10

248	P0CG05	9.43	1	1	106	11.3	7.24	41.5	Ig lambda-2 chain C regions
249	O75390	8.37	1	1	466	51.7	8.32	41.27	Citrate synthase, mitochondrial
250	P26358	0.5	2	1	1616	183	7.75	41.23	DNA (cytosine-5)-methyltransferase 1
251	P48454	1.95	2	1	512	58.1	6.98	40.96	Serine/threonine-protein phosphatase 2B catalytic subunit gamma isoform
252	P58546	17.8	1	1	118	12.9	5.52	40.54	Myotrophin
253	O14818	5.65	1	1	248	27.9	8.46	39.45	Proteasome subunit alpha type-7
254	P05091	1.93	1	1	517	56.3	7.05	39.3	Aldehyde dehydrogenase, mitochondrial
255	Q16698	3.88	1	1	335	36	9.28	39.25	2,4-dienoyl-CoA reductase, mitochondrial
256	P02794	6.01	1	1	183	21.2	5.55	38.97	Ferritin heavy chain
257	Q9NZM1	0.63	1	1	2061	234.6	6.18	37.33	Myoferlin
258	P15153	7.29	1	1	192	21.4	7.61	37.2	Ras-related C3 botulinum toxin substrate 2
259	P62826	6.48	1	1	216	24.4	7.49	37.17	GTP-binding nuclear protein Ran
260	O43852	3.49	1	1	315	37.1	4.64	37.07	Calumenin
261	P00352	2	1	1	501	54.8	6.73	37.06	Retinal dehydrogenase 1
262	Q9H299	10.75	1	1	93	10.4	4.93	36.65	SH3 domain-binding glutamic acid-rich-like protein 3
263	P04040	3.61	1	1	527	59.7	7.39	36.62	Catalase
264	P49902	1.25	1	1	561	64.9	6.14	36.51	Cytosolic purine 5'-nucleotidase
265	O15217	3.6	1	1	222	25.7	8.27	36.31	Glutathione S-transferase A4
266	P51991	2.91	1	1	378	39.6	9.01	35.99	Heterogeneous nuclear ribonucleoprotein A3
267	Q86V81	4.28	1	1	257	26.9	11.15	35.94	THO complex subunit 4
268	Q9Y4G6	0.43	1	1	2542	271.4	5.57	35.5	Talin-2
269	P59998	4.76	1	1	168	19.7	8.43	35.47	Actin-related protein 2/3 complex subunit 4
270	P30038	3.2	1	1	563	61.7	8.07	34.84	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial
271	Q9NYU2	0.84	1	1	1555	177.1	5.63	34.43	UDP-glucose:glycoprotein glucosyltransferase 1
272	Q9UBE0	3.18	1	1	346	38.4	5.3	34.13	SUMO-activating enzyme subunit 1
273	O14556	3.68	1	1	408	44.5	8.19	33.22	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific
274	Q9H0R4	5.02	1	1	259	28.5	6.24	33.1	Haloacid dehalogenase-like hydrolase domain-containing protein 2
275	Q9UN36	4.04	1	1	371	40.8	5.21	32.6	Protein NDRG2
276	Q14624	0.97	1	1	930	103.3	6.98	32.56	Inter-alpha-trypsin inhibitor heavy chain H4
277	O75531	26.97	1	1	89	10.1	6.09	32.41	Barrier-to-autointegration factor
278	P13639	1.4	1	1	858	95.3	6.83	32.03	Elongation factor 2
279	Q9NVE7	1.68	1	1	773	85.9	6.28	31.69	Pantothenate kinase 4
280	Q00610	0.78	1	1	1675	191.5	5.69	31.65	Clathrin heavy chain 1
281	O43432	0.63	1	1	1585	176.5	5.38	31.47	Eukaryotic translation initiation factor 4 gamma 3
282	P68104	2.6	1	1	462	50.1	9.01	31.34	Elongation factor 1-alpha 1
283	Q9NS87	0.72	1	1	1388	160.1	6	31.23	Kinesin-like protein KIF15
284	P30048	4.3	1	1	256	27.7	7.78	31.17	Thioredoxin-dependent peroxide reductase, mitochondrial
285	P30086	7.49	1	1	187	21	7.53	31.04	Phosphatidylethanolamine-binding protein 1

286	P16519	1.25	1	1	638	70.5	6.49	30.96	Neuroendocrine convertase 2
287	Q08211	1.02	1	1	1270	140.9	6.84	30.84	ATP-dependent RNA helicase A
288	P62310	11.76	1	1	102	11.8	4.7	30.38	U6 snRNA-associated Sm-like protein LSm3
289	P37802	3.52	1	1	199	22.4	8.25	29.8	Transgelin-2
290	P12955	2.03	1	1	493	54.5	6	29.19	Xaa-Pro dipeptidase
291	Q13216	2.02	1	1	396	44	6.35	26.81	DNA excision repair protein ERCC-8
292	Q76L83	0.77	1	1	1435	153.7	8.81	26.08	Putative Polycomb group protein ASXL2
293	P55201	0.66	1	1	1214	137.4	7.93	25.7	Peregrin
294	Q07075	0.94	1	1	957	109.2	5.47	25.56	Glutamyl aminopeptidase
295	Q9UPN3	0.12	1	1	7388	837.8	5.39	25.56	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5
296	Q9Y262	1.24	1	1	564	66.7	6.34	24.7	Eukaryotic translation initiation factor 3 subunit L
297	Q14019	6.34	1	1	142	15.9	5.67	24.13	Coactosin-like protein
298	Q9UHN1	1.44	1	1	485	54.9	8.35	21.28	DNA polymerase subunit gamma-2, mitochondrial
299	Q96RL7	0.22	1	1	3174	360	6.33	21.08	Vacuolar protein sorting-associated protein 13A
300	P11117	2.6	1	1	423	48.3	6.74	20.74	Lysosomal acid phosphatase
301	Q9H2C2	3.32	1	1	271	31	8.28	20.31	Protein ARV1

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