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Evaluation of different types of milk proteins-derived epitopes using *in-silico* tools: a primarily study to propose a new definition for bioactive peptides

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Abstract

Bioactive peptides are digestion-resistant and absorbable peptides released from dietary parent proteins. Dietary epitopes exert their effects on the body without absorption and release from parent protein. In this study, the epitope content of milk proteins was discovered, and a new definition for BPs is provided alongside the classic definition. In this study, LBtope, ABCpred, and SVMTriP servers were used to find Linear B cell epitopes. Besides, to predict T cell epitopes, the major histocompatibility complex I (MHC-I) and MHC-II alleles that were more abundant among Iranian people were first found from the Allele Frequency Net Database (AFND). Consequently, the IEDB, RANKPEP, SYFPEITHI, EpiJen, and EpiTOP3 servers were used for MHC binding epitopes. In the current study, MHC-II, MHC-I, and B-cell epitopes of milk proteins were discovered. The most important B-cell epitopes discovered by LBtope, ABCpred, and SVMtrip databases for α_{S1}-casein included APSFSDIPNPIGSEN (176-190), AESISSSEEIVPNSVE (62-77), and VFGKEKVNELSKDIGS (31-46). The high rank of α_{S1}-casein derived from MHC-II epitopes and discovered from the SYFPEITHI database included KEKVNELSKDIGSES (34-48), RFFVAPFPEVFGKEK (22-36), and PELFRQFYQLDAYPS (147-161). The epitopes of dietary proteins and endogenous proteins could be considered as BPs. Epitopes exert their biological effects without absorption and release from parent proteins.

Keywords: milk proteins; bioactive peptides; epitopes; in silico; characterization.

Practical Application: The oral-administered epitopes exert their effects on the body system without absorption and release from parent protein.

1 Introduction

During the last four decades, several investigations have been conducted to assess the properties and physiological effects of bioactive peptides (BPs) and the definition of BPs have been formed with three major characteristics, including **a)** digestion-resistant, **b)** absorbable peptides released from the parent proteins during digestion processes, and **c)** induce a series of beneficial effects on the body (Ji et al., 2021; Rafiq et al., 2021; Shori et al., 2020l ; Shi & Li, 2021). BPs are food-derived peptides whose usefulness is beyond the supply of the body amino-acids (Barati, 2020b; Büyükcan & Karakaya, 2021). Anti-inflammatory, anti-cancer and, anti-hypertensive roles are some of the beneficial effects of the peptides on the body systems (Barati et al., 2020a; Navarro-Peraza et al., 2020). While the absorbability and digestion-resistance are inevitably required for a peptide to exert its effects. Although the definition is not wrong, it covers a limited part of reality. The dietary proteins-derived epitopes are encrypted peptide fragments that do not necessarily meet the absorbability and digestion resistance conditions. However,

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given their function in the gastro-intestinal tract (GIT) should be included in the definition to provide a comprehensive definition of BPs (Li et al., 2021; Zhou et al., 2021).

When dietary proteins enter into the intestine, the gut immune system, named gut-associated lymphoid tissue (GALT), takes the GIT content and assesses the proteins. During the assessment of dietary proteins by GALT, several active fragments in the parent proteins are identified, and after identification of the active encrypted peptide fragments, the GALT begins to suppress any immune responses against the fragments in the body. The process in which the immune response against the dietary active peptide fragments is suppressed is called oral tolerance, and the active encrypted peptide fragments are known as epitopes (Barati et al., 2020a; Pabst & Mowat, 2012). In physiological conditions, the body's immune system tolerates all of the dietary proteins. Nevertheless, in some pathological conditions such as celiac disease and food allergy, the oral tolerance against dietary proteins is incomplete and immune response against the GIT contents makes several signs and symptoms that these symptoms can be life-threatening in some cases (Meresse et al., 2009; Satitsuksanoa et al., 2018). For instance, in celiac disease, oral tolerance against gluten does not occur; therefore, the GALT response against gut contents contributed to a pathological condition (Parzanese et al., 2017; Şirin & Yalçın, 2019).

As mentioned above, dietary epitopes (exogenous epitopes) are peptide fragments encrypted in dietary proteins. The epitopes exert their bioactivity without intestinal absorption or release from parent proteins. GALT samples GIT contents and finds epitopes in the proteins, suppressing the immune response against their active fragments. It should be noted that each protein has its epitopes, either exogenous proteins (dietary proteins) or endogenous proteins (the body's proteins) (Pabst & Mowat, 2012; Takahashi et al., 2019). Endogenous and exogenous epitopes can be divided into 1) B-cell epitopes, 2) MHC-I epitopes, and 3) MHC-II epitopes. Each epitope has its fate in GIT and exerts different biological effects on the body systems (Palatnik-de-Sousa et al., 2018).

In an autoimmune disease such as multiple sclerosis and lupus, the immune system responds to endogenous epitopes (Liao et al., 2016). In recent years, studies have identified that endogenous epitopes caused autoimmune disease, and oral administration of synthetic forms of these epitopes in a proper form provided a possible way to treat these types of diseases (Mundkur et al., 2013; Thota et al., 2017). On the other hand, in a novel perspective, many studies have been conducted to treat food allergies by oral administration of the epitopes of same foods. In these kinds of studies, the epitopes of food allergens are predicted, synthesized and orally introduced to the experimental food allergy model. Therefore, predictions of food-derived epitopes are very important to treat allergies. It should be noted the orally administered fragments (poly-epitopes) are not digestion resistant and do not absorb in their intact form. If epitopes consider (either endogenous or exogenous) as bioactive fragments, digestion resistance and absorbability do not necessarily need to be included in the BPs definition (Barra et al., 2020).

Considering the importance of the determination of food epitopes, in the current work, the epitopes of cow's milk proteins are predicted. Cow's milk allergy is one of the most common allergies around the world and determination of its epitopes have pivotal role in treatment of the disease by the novel perspective. On the other hand, in the current work, a novel and comprehensive definition for BPs is suggested to cover the epitopes' nature and function. In fact, in this study, peptide-fragments have been reported that are not digestible and absorbable, but can exert their biological effects through the immune system.

2 Material and methods

2.1 Prediction of linear B cell epitopes

The α_{s1}-casein (P02662|16-214), α_{s2}-casein (P02663|16-222), β-casein (P02666|16-224), k-casein (P02668|22-190), β-lactoglobulin (P02754|17-178) and α-lactalbumin (P00711|20-142), the major contributors of milk proteins, were obtained from UniProtKB (https://www.uniprot.org) database (Supplementary Table 1). The intact forms of the proteins were used to predict their epitopes.

Linear B cell epitopes were obtained using LBtope (https://webs.iiitd.edu.in/raghava/lbtope/), ABCpred (https://webs.iiitd.edu.in/raghava/abcpred/), and SVMTriP servers (http://sysbio.unl.edu/SVMTriP/). The length of selected epitopes were 15 and 16 amino acids in ABCpred and SVMTriP, respectively, and LBtope variable lengths were selected. These servers are developed based on different methods and algorithms. The overlapping sequences are common between at least two servers. Therefore, these overlapping were considered the optimal linear B cell dominant epitope of the proteins, which can considerably improve epitope prediction reliability.

2.2 Prediction of T cell epitope

We obtained the highest frequency alleles in Iran from the AFND database (http://www.allelefrequencies. net/hla.asp), including HLA-A*02, HLA-B*35, HLA-A*24, HLA-B*51, HLA-A*01, and HLA-A*03 recognized by MHC-I. Also, HLA-DRB1*11, HLA-DRB1*15, HLA-DRB1*04, HLA-DRB1*13, and HLA- DQA1*05-DQB1*03:01 recognized by MHC-II.

MHC-I binding epitopes were predicted by IEDB MHC I tool (http://tools.immuneepitope.org/mhci/), EpiJen (http://www.ddg-pharmfac.net/epijen/EpiJen/EpiJen.htm), RANKPEP (http://imed.med.ucm.es/Tools/rankpep .html), and SYFPEITHI (http://www.syfpeithi.de/bin/mhcserver. dll/epitopeprediction.htm). IEDB MHC I Consensus method, which combines ANN, SMM, and Comblib_Sidney2008, was used. 9-mer epitope lengths were selected. MHC-II binding epitopes were predicted by the IEDB MHC-II tool (http://tools.immuneepitope.org/mhcii/), EpiTOP3 (http://www.ddg-pharmfac.net/EpiTOP3/), RANKPEP, and SYFPEITHI. IEDB MHC II Consensus method, which combines NN-align, SMM-align, CombLib, and Sturniolo, was used. 15-mer epitope lengths were selected. The overlapping sequences that occur in the results of three or four servers were defined as the optimal MHC-I or MHC-II binding dominant epitope of the proteins. It should be noted that the SYFPEITHI

does not yet provide analysis about DRB1*13 and HLA- DQA1*05-DQB1*03:01. Also, RANKPEP does not yet provide an analysis about HLA- DQA1*05-DQB1*03:01.

Some servers have predicted output as units of IC50nM or -logIC50 M (pIC50). The lower IC50nM value or higher -logIC50 M value indicates the higher affinity. Peptides with IC50 values <50 nM are usually considered to have high affinity. The predicted output in the IEDB server is percentile rank, which is obtained by comparing the IC50 values of the various methods used. A small numbered percentile rank suggests high affinity. In the RANKPEP server, in addition to the score, it also gives % OPT (optimal score), which is the percentile score of the peptide concerning the consensus (a sequence that yields the maximum score).

3 Results

The current study was designed to discover different epitopes, including B-cell, MHC-I, and MHC-II types in the milk proteins. The most critical B-cell epitopes discovered by LBtope, ABCpred, and SVMtrip databases for α_{S1}-casein were APSFSDIPNPIGSEN (176-190), AESISSSEEIVPNSVE (62-77), and VFGKEKVNELSKDIGS (31-46). The top 10 B-cell epitopes, in terms of retrieved score, were discovered by each database for milk proteins (Table 1). When ABCpred was used, some of the epitopes gain an equal score. For instance, analysis of α_{S2}-casein sequence by ABCpred database showed EESAEVATEEVKITVD (59-74), QEKNMAINPSKENLCS (22-37), KTVYQHQKAMKPWIQP (186-196), and DMESTEVFTKKTKLTE (140-155) as B-cell epitopes with an equal score. B-cell epitope analysis of milk proteins showed κ-casein, β-casein, α-lactalbumin, β-lactoglobulin, α_{S1}-casein, and α_{S2}-casein contain 28, 36, 29, 28, 28, and 33 epitopes, respectively. Therefore, β-casein and α_{S2}-casein have more B-cell epitope capacity. The databases may report similar epitopes such as β-casein derived epitopes, including EDELQDKIHPFAQTQ (42-56; score: 1.47) EDELQDKIHPFAQTQS (42-57; score: 0.76) were achieved by LBtope and ABCpred databases, respectively.

IEDB, RankPep, EpiJen, and SYFPEITHI databases were used for discovering milk-derived MHC-I epitopes. The discovered MHC-I epitopes are summarized in Supplementary Table 2. For each database, HLA-A*02:01, HLA-A*24:02, HLA-A*01:01, HLA-A*03:01, HLA-B*35:01, and HLA-B*51:01 were considered as alleles. Κ-casein derived MHC-I epitopes such as ATLEDSPEV (144-152), KYIPIQYVL (24-32), LINNQFLPY (50-58), VLSNTVPAK (78-86), YPSYGLNYY (35-43), and HPHLSFMAI (100-108) are high-rank IEDB discovered epitopes achieved across the mentioned alleles, respectively.

For identification of milk-derived MHC-II epitopes, IEDB, Rankpep, EpiTOP 3.0, and SYFPEITHI databases and alleles, including HLA-DRB1*11:01, HLA-DRB1*15:01, HLA-DRB1*04:01, HLA-DRB1*13:02, and HLA-DQA1*05:01/DQB1*03:01 were used. Rankpep database does not cover HLA-DQA1*05:01/DQB1*03:01. Therefore, milk-derived MHC-II epitopes across the allele from Rankpep were not reported. Due to the same reason, the epitopes from the SYFPEITHI database were not reported across HLA-DRB1*13:02 and HLA-DQA1*05:01/DQB1*03:01. The discovered MHC-II epitopes are summarized in Supplementary Table 3. The high-rank α_{S1}-casein derived MHC-II epitopes discovered from the SYFPEITHI database based on HLA-DRB1*11:01, HLA-DRB1*15:01 and HLA-DRB1*04:01 included KEKVNELSKDIGSES (34-48), RFFVAPFPEVFGKEK (22-36), and PELFRQFYQLDAYPS (147-161), respectively.

4 Discussion

Each of the epitopes, including MHC-I, MHC-II, and B-cell epitopes, has its own fate in the GIT. When dietary proteins enter the intestine, the gut immune system continuously samples the lumen content and analyses it immunologically. GALT assesses the food content of the lumen as a graft. In the most time, the immune response against the gut content is suppressed (Barati et al., 2020b). However, sometimes immune responses occur. A well-known example of this situation is the pathologic immune response seen in celiac disease against the food content after intake of wheat proteins (Parzanese et al., 2017). Milk allergy is another example of the immune response to gut content (Verhasselt, 2010). It is out of this manuscript's scope to further discuss the GALT response to gut content during pathologic circumstances and its pathogenesis. Conversely, the more specific aspect of the process that suppresses immune responses against the gut content is elaborated in this study. It seems some forms of immune system cells, including regulatory B and regulatory T cells, have an important role in this process.

As mentioned, gut content is sampled continuously by GALT. Also, the food proteins can be diffused into the submucosa and directly be exposed to the immune system's cells (Kostovcikova et al., 2019). Although in this *in-silico* study we identified the MHC-I, MHC-II, and B-cell epitopes, it is believed that the MHC-II and B-cell epitopes are biologically important in GIT (Carrera et al., 2019; He et al., 2013; Mundkur et al., 2013; Thota et al., 2017). MHC-II epitopes are identified by a type of immune system cells called dendritic cells (DCs). After identifying the epitopes by DCs, the cells migrate to the lymph nodes and interact with other immune cells called T cells that are also called naïve in this stage of maturation and indicate an absence of the previous encounter with the antigen. Interaction of DCs with naïve T cells in the gut typically contributes to deleting the cell known as clonal deletion or differentiation of the naïve T cells into regulatory T cells (T-regs). If a specific T-reg colony expands, it can potentially suppress immune responses against the body's corresponded epitope (Osorio et al., 2015). The exact process also activates in the exposure of dietary B-cell epitopes to GALT, and a kind of immune cell called regulatory B cells differentiate from naïve or other B cells. This kind of B-cells exactly acts as an immunosuppressant against dietary B-cell epitopes (Doherty et al., 2018; Kim et al., 2016). Therefore, Regulatory T and B cells have pivotal roles in suppressing immune responses against gut content. This process is called oral tolerance (Pabst & Mowat, 2012). The mechanisms related to the T-regs and oral tolerance against MHC-II epitopes are widely elucidated, but the exact mechanisms for expanding B-regs are not adequately known yet. However, it is believed that both regulatory cells' formation is interdependent (Doherty et al., 2018; Kim et al., 2016). Considering what was reported in this section, it seems that oral administration of proteins can induce specific oral tolerance

Table 1. The linear B-cell epitopes obtained from in-silico analysis of milk proteins in LBtope, ABCpred and SVMtrip databases.

	LBtope				ABCpred				SVMtrip			
	Rank	Location	Epitope	Score	Rank	Location	Epitope	Score	Rank	Location	Epitope	Score
α_{S1}-casein	1	176-190	APSFSDIPNPIGSEN	1.4268331	1	62-77	AESISSSEEIVPNSVE	0.88	1	31 - 46	VFGKEKVNELSKDIGS	1.000
	2	13-27	QEVLNENLLRFFVAP	1.4218801	2	3-18	KHPIKHQGLPQEVLNE	0.87	2	89 - 104	ERYLGYLEQLLRLKKY	0.990
	3	84-98	EDVPSERYLGYLEQL	1.4115688	3	159-174	YPSGAWYYVPLGTQYT	0.85	3	109 - 124	LEIVPNSAEERLHSMK	0.871
	4	14-28	EVLNENLLRFFVAPF	1.4049493	4	171-186	TQYTDAPSFSDIPNPI	0.82	4	12 - 27	PQEVLNENLLRFFVAP	0.829
	5	83-97	KEDVPSERYLGYLEQ	1.3760909	5	107-122	PQLEIVPNSAEERLHS	0.81	5	53 - 68	AMEDIKQMEAESISSS	0.600
	6	17-31	NENLLRFFVAPFPEV	1.3584404	6	42-57	KDIGSESTEDQAMEDI	0.80	6	136 - 151	IGVNQELAYFYPELFR	0.568
	7	24-38	FVAPFPEVFGKEKVN	1.3489913	6	141-156	ELAYFYPELFRQFYQL	0.80	7	184 - 199	NPIGSENSEKTTMPLW	0.509
	8	31-45	VFGKEKVNELSKDIG	1.345489	7	177-192	PSFSDIPNPIGSENSE	0.79	8	154 - 169	YQLDAYPSGAWYYVPL	0.490
	9	16-30	LNENLLRFFVAPFPE	1.328342	8	78-93	QKHIQKEDVPSERYLG	0.77				
	10	26-40	APFPEVFGKEKVNEL	1.3225096	8	69-84	EEIVPNSVEQKHIQKE	0.77				
α_{S2}-casein	1	83-97	NEINQFYQKFPQYLQ	1.474101	1	11-26	EESIISQETYKQEKNM	0.96	1	32 - 47	KENLCSTFCKEVVRNA	1.000
	2	42-56	EVVRNANEEEYSIGS	1.3732227	2	51-66	EYSIGSSSEESAEVAT	0.91	2	70 - 85	KITVDDKHYQKALNEI	0.946
	3	84-98	EINQFYQKFPQYLQY	1.3575592	3	93-108	PQYLQYLYQGPIVLNP	0.88	3	110 - 125	DQVKRNAVPITPTLNR	0.429
	4	92-106	FPQYLQYLYQGPIVL	1.3305821	4	124-139	NREQLSTSEENSKKTV	0.86	4	157 - 172	EKNRLNFLKKISQRYQ	0.409
	5	90-104	QKFPQYLQYLYQGPI	1.1269925	5	68-83	EVKITVDDKHYQKALN	0.84	5	133 - 148	ENSKKTVDMESTEVFT	0.385
	6	43-57	VVRNANEEEYSIGSS	1.1168358	5	30-45	PSKENLCSTFCKEVVR	0.84	6	11 - 26	EESIISQETYKQEKNM	0.362
	7	30-44	PSKENLCSTFCKEVV	1.095805	6	101-116	QGPIVLNPWDQVKRNA	0.83	7	177 - 192	PQYLKTVYQHQKAMKP	0.361
	8	91-105	KFPQYLQYLYQGPIV	1.0780446	7	116-131	AVPITPTLNREQLSTS	0.82	8	49 - 64	EEEYSIGSSSEESAEV	0.361
	9	93-107	PQYLQYLYQGPIVLN	1.0680734	8	152-167	KLTEEEKNRLNFLKKI	0.79				
	10	41-55	KEVVRNANEEEYSIG	1.0645073	9	59-74	EESAEVATEEVKITVD	0.78				
					9	22-37	QEKNMAINPSKENLCS	0.78				
					9	181-196	KTVYQHQKAMKPWIQP	0.78				
					9	140-155	DMESTEVFTKKTKLTE	0.78				
					10	4-19	MEHVSSSEESIISQET	0.76				
					10	130-145	TSEENSKKTVDMESTE	0.76				
β-lactoglobulin	1	13-27	QKVAGTWYSLAMAAS	0.87882212	1	120-135	QCLVRTPEVDDEALEK	0.84	1	20 - 35	YSLAMAASDISLLDAQ	1.000
	2	46-60	LKPTPEGDLEILLQK	0.87572925	2	80-95	AVFKIDALNENKVLVL	0.83	2	129 - 144	DDEALEKFDKALKALP	0.567
	3	103-117	LLFCMENSAEPEQSL	0.80592904	3	14-29	KVAGTWYSLAMAASDI	0.78	3	81 - 96	VFKIDALNENKVLVLD	0.343
	4	149-163	LSFNPTQLEEQCHIX	0.79690354	4	127-142	EVDDEALEKFDKALKA	0.77	4	98 - 113	DYKKYLLFCMENSAEP	0.238
	5	148-162	RLSFNPTQLEEQCHI	0.75199951	5	53-68	DLEILLQKWENGECAQ	0.75				
	6	125-139	TPEVDDEALEKFDKA	0.71099026	6	62-77	ENGECAQKKIIAEKTK	0.72				
	7	123-137	VRTPEVDDEALEKFD	0.64474376	6	41-56	VYVEELKPTPEGDLEI	0.72				
	8	75-89	KTKIPAVFKIDALNE	0.61451937	7	23-38	AMAASDISLLDAQSAP	0.71				
	9	25-39	AASDISLLDAQSAPL	0.6042067	7	106-121	CMENSAEPEQSLACQC	0.71				
	10	102-116	YLLFCMENSAEPEQS	0.56811264	8	68-83	QKKIIAEKTKIPAVFK	0.67				
					9	2-17	IVTQTMKGLDIQKVAG	0.62				
					9	136-151	FDKALKALPMHIRLSF	0.62				
					10	96-111	DTDYKKYLLFCMENSA	0.61				
					10	90-105	NKVLVLDTDYKKYLLF	0.61				

Alanine: A; Arginine: R; Asparagine: N; Aspartic acid: D; Cysteine: C; Glutamic acid: E; Glutamine: Q; Glycine: G; Histidine: H; Isoleucine: I; Leucine: L; Lysine: K; Methionine: M; Phenylalanine: F; Proline: P; Serine: S; Threonine: T; Tryptophan: W; Tyrosine: Y; Valine: V.

Table 1. Continued...

	LBtope				ABCpred				SVMtrip			
	Rank	Location	Epitope	Score	Rank	Location	Epitope	Score	Rank	Location	Epitope	Score
α-lactalbumin	1	95-109	ILDKVGINYWLAHKA	1.3981854	1	98-113	KVGINYWLAHKALCSE	0.9	1	104 - 119	WLAHKALCSEKLDQWL	1.000
	2	71-85	NICNISCDKFLDDDL	1.2032195	2	52-67	LFQINNKIWCKDDQNP	0.88	2	83 - 98	DDLTDDIMCVKKILDK	0.890
	3	1-14	XEQLTKCEVFRELKD	1.195677	3	68-83	HSSNICNISCDKFLDD	0.82	3	1 - 16	EQLTKCEVFRELKDLK	0.549
	4	87-101	DDIMCVKKILDKVGI	1.1761769	3	59-74	IWCKDDQNPHSSNICN	0.82	4	40 - 55	AIVQNNDSTEYGLFQI	0.301
	5	92-106	VKKILDKVGINYWLA	1.1690351	3	30-45	TFHTSGYDTQAIVQNN	0.82	5	57 - 72	NKIWCKDDQNPHSSNI	0.247
	6	4-18	TKCEVFRELKDLKGY	1.1585111	4	78-93	DKFLDDDLTDDIMCVK	0.78	6	19 - 34	GGVSLPEWVCTTFHTS	0.229
	7	91-105	CVKKILDKVGINYWL	1.1566838	5	10-25	RELKDLKGYGGVSLPE	0.76				
	8	94-108	KILDKVGINYWLAHK	1.1211054	6	23-38	LPEWVCTTFHTSGYDT	0.75				
	9	6-20	CEVFRELKDLKGYGG	1.1024283	7	44-59	NNDSTEYGLFQINNKI	0.67				
	10	7-21	EVFRELKDLKGYGGV	1.0963599	8	16-31	KGYGGVSLPEWVCTTF	0.66				
					9	38-53	TQAIVQNNDSTEYGLF	0.65				
					10	103-88	DIMCVKKILDKVGINY	0.6				
					10	106-121	AHKALCSEKLDQWLCE	0.6				
β-casein	1	42-56	EDELQDKIHPFAQTQ	1.4720084	1	9-24	PGEIVESLSSSEESIT	0.87	1	3 - 18	LEELNVPGEIVESLSS	1.000
	2	193-207	YQEPVLGPVRGPFPI	1.442218	2	75-90	PPLTQTPVVVPPFLQP	0.86	2	127 - 142	LTDVENLHLPLPLLQS	0.857
	3	194-208	QEPVLGPVRGPFPII	1.4222002	3	152-167	PPTVMFPPQSVLSLSQ	0.84	3	90 - 105	PEVMGVSKVKEAMAPK	0.640
	4	40-54	QTEDELQDKIHPFAQ	1.4167443	3	109-124	MPFPKYPVEPFTESQS	0.84	4	20 - 35	EESITRINKKIEKFQS	0.595
	5	41-55	TEDELQDKIHPFAQT	1.4167443	3	100-115	EAMAPKHKEMPFPKYP	0.84	5	181 - 196	PQRDMPIQAFLLYQEP	0.590
	6	171-185	LPVPQKAVPYPQRDM	1.3999914	4	16-31	LSSSEESITRINKKIE	0.80	6	162 - 177	VLSLSQSKVLPVPQKA	0.590
	7	192-206	LYQEPVLGPVRGPFP	1.3902037	5	64-79	GPIPNSLPQNIPPLTQ	0.78	7	45 - 60	LQDKIHPFAQTQSLVY	0.589
	8	1-15	RELEELNVPGEIVES	1.3760173	5	33-48	FQSEEQQQTEDELQDK	0.78	8	71 - 86	PQNIPPLTQTPVVVPP	0.589
	9	4-18	EELNVPGEIVESLSS	1.367274	5	190-205	FLLYQEPVLGPVRGPF	0.78				
	10	39-53	QQTEDELQDKIHPFA	1.3583242	5	138-153	PLLQSWMHQPHQPLPP	0.78				
					5	120-135	TESQSLTLTDVENLHL	0.78				
					6	144-159	MHQPHQPLPPTVMFPP	0.77				
					7	42-57	EDELQDKIHPFAQTQS	0.76				
					7	3-18	LEELNVPGEIVESLSS	0.76				
					8	181-196	PQRDMPIQAFLLYQEP	0.75				
					9	56-71	QSLVYPFPGPIPNSLP	0.74				
					9	166-181	SQSKVLPVPQKAVPYP	0.74				
					10	27-42	NKKIEKFQSEEQQQTE	0.69				
κ-casein	1	150-164	PEVIESPPEINTVQV	1.7472975	1	38-53	YGLNYYQQKPVALINN	0.91	1	13 - 28	KDERFFSDKIAKYIPI	1.000
	2	93-107	TTMARHPHPHLSFMA	1.6720176	2	128-143	GEPTSTPTTEAVESTV	0.89	2	141 - 156	STVATLEDSPEVIESP	0.895
	3	152-166	VIESPPEINTVQVTS	1.6198306	2	101-116	PHLSFMAIPPKKNQDK	0.89	3	112 - 127	KNQDKTEIPTINTIAS	0.568
	4	101-115	PHLSFMAIPPKKNQD	1.5215897	3	30-45	YVLSRYPSYGLNYYQQ	0.88	4	94 - 109	TMARHPHPHLSFMAIP	0.518
	5	151-165	EVIESPPEINTVQVT	1.5055497	4	150-165	PEVIESPPEINTVQVT	0.87	5	61 - 76	YAKPAAVRSPAQILQW	0.467
	6	11-25	CEKDERFFSDKIAKY	1.5051713	5	90-105	AQPTTMARHPHPHLSF	0.86	6	36 - 51	PSYGLNYYQQKPVALI	0.466
	7	149-163	SPEVIESPPEINTVQ	1.4581587	6	119-134	IPTINTIASGEPTSTP	0.78				
	8	6-20	EQPIRCEKDERFFSD	1.4313729	7	10-25	RCEKDERFFSDKIAKY	0.76				
	9	94-108	TMARHPHPHLSFMAI	1.4260776	8	135-150	TTEAVESTVATLEDSP	0.74				
	10	100-114	HPHLSFMAIPPKKNQ	1.4189371	9	58-73	YPYYAKPAAVRSPAQI	0.73				
					9	110-125	PKKNQDKTEIPTINTI	0.73				
					10	141-156	STVATLEDSPEVIESP	0.70				

Alanine: A; Arginine: R; Asparagine: N; Aspartic acid: D; Cysteine: C; Glutamic acid: E; Glutamine: Q; Glycine: G; Histidine: H; Isoleucine: I; Leucine: L; Lysine: K; Methionine: M; Phenylalanine: F; Proline: P; Serine: S; Threonine: T; Tryptophan: W; Tyrosine: Y; Valine: V.

against the proteins. The immune response against the own body protein makes autoimmune diseases such as cardiovascular disease (CVD) (Mundkur et al., 2013; Thota et al., 2017), multiple sclerosis (MS) (Wootla et al., 2012). Oral administration of the proteins involved in the immunopathogenesis of autoimmune diseases can theoretically induce tolerance against the targets and can be considered a potential relief to the disease. On the other hand, there are many studies evaluated the effects of oral administration of epitopes to treat food allergy. Candreva et al. in an experimental study showed that oral administration of an epitope-containing soy peptide significantly ameliorates experimental milk allergy (Candreva et al., 2021). In a similar work, Rupa et al. revealed that oral administration of T-cell epitopes of egg-white-ovomucoid significantly improved clinical signs of egg allergy in mice (Rupa & Mine, 2012). Other than the mentioned works, there are many similar studies for allergy ameliorating using oral administration of epitopes (Kawabe et al., 2012; Takagi et al., 2005; Thang & Zhao, 2015). It may come to the minds of readers of this text that proteins and/or epitopes are digested when they enter the digestive tract and cannot provide these activities. This is true, but GALT continuously samples the GIT content before, during and after of digestion; therefore, the immune system has the opportunity to pick-up the intact form of poly-epitopes from the digestive tract (Hoh & Boyd, 2018).

There are many researches on BPs around the world, and the researchers believe that BPs should be released from dietary proteins, be digestion resistant and absorbable to induce their biological effects. In this work, we reported several peptides fragments that are neither digestion resistant, and nor absorbed into the general circulation, but they induced immunomodulatory effects on the body when introduced orally. Accepting the new definition could strengthen the link between researches on BPs and food-biotechnology and deeper work can be done in this area.

5 Future perspectives

According to our results, two different definitions for BPs could be considered. The first one, the classic definition which defines BPs as digestion-resistant and absorbable peptides released from the parent proteins during digestion processes and induces a series of beneficial effects on the body (Barati et al., 2020b). For doing a scientific project according to the classic definition, several specific steps need to be done, including selecting a food item, isolating the food item protein, digestion of the isolate (by enzymes or microorganisms), characterization of refractory digestion fragments, and evaluation of their function *in-vitro* and/or *in-vivo* (Barati et al., 2020b). According to the classic definition, the studies are widely performed, while the studies on BPs can be expanded according to a different and parallel definition in which dietary epitopes can be considered bioactive peptides. The dietary epitopes are peptide fragments within dietary proteins that exert their biological functions independent of their release from parent proteins or absorption (Pabst & Mowat, 2012).

We comprehensively discussed dietary epitopes in this study. Therefore, if the dietary epitopes considered as bioactive peptides, working on BPs with this new definition opens a new view to design studies to determine the epitopes that make disease in the body, synthesis of the epitopes, and oral administration of the synthesized epitopes to experimental models (He et al., 2013; Mundkur et al., 2013; Thota et al., 2017). For instance, the immune response against MHC-II epitopes of endogenous LDL and HSP 70 has a pivotal role in cardiovascular disease pathogenesis (Figure 1). Synthesis of the epitopes of LDL, HSP 70, and oral administration of the epitopes suppress the immune response against the structure and improve cardiovascular disease

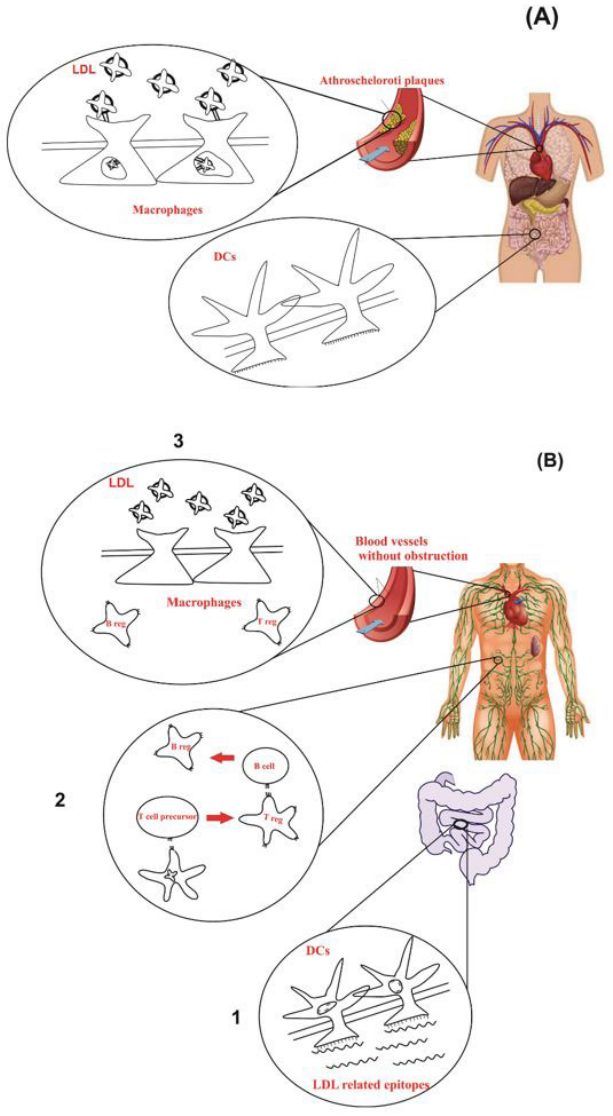

Figure 1. **A)** The macrophages located on vessel epithelium gathering LDLs from general circulation by interacting with LDL related epitopes and make a pathologic condition named atherosclerosis. **B) 1:** LDL related epitopes introduce orally and the epitopes is picked up by dendritic cells (DCs) located in the gut epithelium. **2:** The DCs containing LDL related epitopes migrate to lymph nodes and interact with a kind of undifferentiated T cells named Naïve T cells or T cell precursor. After the interaction, T cell precursors differentiate to regulatory T cells (T-regs). Differentiation in B-cell precursors occur after exposure to T-regs and a kind of B-cells named B-regs form. **3:** The T-regs and B-regs migrate to vessel epithelium and suppress any immune response against LDL related epitopes and improve arteriosclerotic plaques.

(Mundkur et al., 2013; Thota et al., 2017). Mundkur et al., in an experimental study, orally administered MHC-II epitopes of LDL and HSP 70 in mice with cardiovascular disease. The study results demonstrated that this intervention declines cardiovascular disease progression in the mice (Mundkur et al., 2013). In a similar study, Thota et al. reported similar results (Thota et al., 2017). In the both mentioned experimental studies, the pathogenic epitopes exposed to GALT directly; While, indirect exposure of the epitopes using engineered-probiotics could enhance the bioactivity of the fragments (Zhou et al., 2020).

Few studies were done to clarify the effects of orally administered epitopes on the initiation and progression of immune system-related diseases such as cardiovascular disease and MS. The suggestion of a new definition for BPs can dramatically expand researchers' perspectives in the field of bioactive peptides.

6 Conclusion

In this study, different types of dairy protein epitopes have been identified, and the exact fate of each type of epitope is discussed. Also, a novel definition for bioactive peptides in parallel with the classical definition is introduced. In the newly proposed definition, dietary epitopes are introduced as BPs. Therefore, the newly defined pathway pushes forward the studies on BPs to a new view that previously has not been explored. It is undeniable that both of the definitions have their benefits and limitation.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Material

Supplementary material accompanies this paper.

Supplementary Table 1. The amino-acids sequence of bovine's milk proteins.

Supplementary Table 2. MHC-I epitope content of bovine's milk protein.

Supplementary Table 3. MHC-II epitope content of bovine's milk protein.

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