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Pattern Similarity Analysis of Amino Acid Sequences for Peptide Emulsification

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A new computer program for homology similarity search (HSS) was introduced. Application of the HSS to peptide sequences of short peptides with fewer 32 amino acid residues has explained the underlying mechanism of their emulsifying ability. It was found that certain regularity in the frequency of alternate polar/apolar cycle with high hydrophobic similarity density was required to obtain good emulsion. To supplement this required regularity, charge distribution, molecular flexibility, and a structural torsion caused by a proline residue might also play roles.

KEYWORDS: Homology similarity search; peptide emulsion; pattern similarity; amino acid sequence; QSAR; hydrophobicity distribution

INTRODUCTION

Pioneering work of quantitative structure−activity relationships (QSAR) was published by Hellberg et al. (*1*) using the 3z method for modeling the functional behavior of peptides as a function of the physicochemical properties of amino acid composition. The partial least-squares (PLS) method was used for regression analysis of their data. Strøm et al. (*2*) applied the 3z method to the prediction of minimum inhibitory concentration (MIC) of 19 peptides of 15-residue murine lactoferricin (LFcin) and its derivatives. We (*3*) have introduced new software, namely, homology similarity analysis (HSA), to predict the MIC of 66 LFcin derivatives. Artificial neural networks (ANN) were used for regression analysis of our data. It was found that the pattern similarity of segments within sequences, rather than the sequence as a whole, could be a useful tool for representing the distribution attributes of amino acid residue properties to the peptide QSAR. Our work ($R^2 = 0.90$ for predicted/observed, $n = 66$) was superior to that of Strøm et al. (*2*) [$R^2 = 0.60$, $n = 20$, according to Lijon et al. (*4*)] despite the variation of MIC data between the different laboratories in our case. A reason may be the elimination of exceptional 5 peptides from the original 71 peptides. ANN is more flexible, thus being suitable for nonlinear fitting, than PLS (*5*, *6*). Cronin and Schultz (*7*) compared multiple linear regression analysis, PLS regression, and regression ANN for the same toxicological data, which were further supportive of the superiority of ANN.

Outstanding work on peptide emulsification was reported by Saito et al. (*8*, *9*). Using three 16-residue synthetic peptides composed of leucine (L) and glutamic acid (E) alone, the importance of secondary structure was suggested in elucidating their emulsification ability. Huang et al. (*10*) stated that ideal emulsifying properties of a protein or oligopeptide were derived from a delicate balance of charge density and net charge, distribution of polar and hydrophobic residues, tertiary structure, and its flexibility. Because this conclusion was deduced from the surface properties of peptides larger in chain length than those used by Saito et al. (*8*), some restraints may be imposed on applying their rule to shorter peptide chains. Although there have been quite many papers with regard to the emulsifying capacity of protein hydrolysates in the literature, especially in the area of pharmacology, only a few papers have reported the relationships with amino acid sequences using separated pure peptide fractions. We were interested in protein sequence analysis to explain the underlying mechanism of functionality after writing software for functional sequence analysis (*3*).

In the recent review of protein QSAR, Giuliani et al. (*11*) stated that hydrophobicity profiles (patterns) rather than hydrophobicity per se were playing extremely important roles in protein functions, in which signal analysis techniques were used to quantify the profiles. They defined the hydrophobicity profile as the distribution of periodicity of hydrophobicity in protein sequences. Therefore, it is possible that our newly introduced software for homology similarity search (HSS) may be useful in elucidating peptide emulsification.

The objective of this study was to apply the HSS to known peptide sequences to provide a reasonable explanation to the underlying mechanism of their emulsification despite the difficulty in comparing emulsifying ability data reported from different laboratories. However, this problem is rather common

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Table 1. Short-Chain Peptides with 10–32 Residues[a]

Peptide	Sequence	Emulsification	Absorption	Reference
1 Peptide S	E L E L E L E L E L E L E L	EAI 61 (0.1%)	Abs(500).372	[8, 9]
2 Peptide H	L E E L L E E L L E E L L E E L	" " " 27 (0.1%)	" " " .157	[8, 9]
3 Peptide R	L E L L E E E L L E E E L L E L	" " " 12 (0.1%)	" " " .071	[8, 9]
4 (O-4632 SIGMA)	Y Q E A F R R F F G P V	" " " 3.5 (0.1%)	Abs(500).020	This study
5 (F-9145 SIGMA)	H H L G G A K Q A G N V	" " " 7.5 (0.1%)	" " " .043	This study
6 (S-7152 SIGMA)	S F L L R N P N N K Y E P F	" " " 10 (0.1%)	" " " .055	This study
7 Salmine	P R R R R S S S R P I R R R R P R R A S R R R R R R G G R R R R	" " " 7 (0.1%)	" " " .04	This study
8 Bovine lactoferricin	F K C R R W Q W R M K K L G A P S I T C V R R A F	" " " 14 (0.1%)	" " " .08	This study
9 betaLg(21-40)	S L A M A A S D I S L L D A N S A P L R		AbsRate ~.8	[18]
10 (41-60)	V Y V E E L K P T P E G D L D I L L Q K		" " " 1.27	[18]
11 (61-70)	W E N G E C A N K K		" " " <.1	[18]
12 (149-162)	L S F N P T Q L E E N C H I		" " " <.1	[18]
13 alpha-s1CN(1-23)	R P K H P I K H Q G L P Q E V L N E N L L R F	" " " 10 (1.0%)		[15]
14 betaCN(1-25)	R E I E E I N V P G E I V E S L S S S E E S I T R	" " " 10 (2.0%)		[16]
15 betaCN(193-209)	Y Q Q P V I G P V R G P F P I I V	" " " 8 (2.0%)		[16]

[a] EAI, emulsification activity index; AbsRate, absorption rate computed from surface tension.

in the case of any data-mining study. Our special interest was in the comparison of the sequences of protamine and bovine lactoferricin as cationic antimicrobial peptides (CAP), because of a difference in the mechanism of their invasion of the bacterial cell matrix (*12*).

MATERIALS AND METHODS

Peptides. Salmine and peptides O-4632 (12 residues), F-9145 (12 residues), and S-7152 (14 residues) were purchased from Sigma, Oakville, ON, Canada. Bovine lactoferricin was obtained through the courtsey of Morinaga Milk Industry Co., Tokyo, Japan.

Lipophilization. Protein−fatty acid condensation using the fatty acid chloride (R−COCl) of Kabanov et al. (*13*) was employed.

Emulsifying Activity. The emulsifying activity index (EAI) was measured according to the method of Saito et al. (*8*) after minor modifications. To 1.4 mL of 0.1% peptide solution in 5 mM phosphate buffer, pH 7.0, containing 0.2 M NaCl was added 0.35 mL of corn oil. The mixture was homogenized in a sonicator (Tekmer sonic disruptor, Cincinnati, OH) for 2 min on 50% duty cycle and pulsed mode. After homogenization, the turbidity of a 10 μL aliquot in 8.6 mL of 0.1% sodium dodecyl sulfate solution was measured at 500 nm. The EAI was reported in m^2/g.

Homology Similarity Analysis. HSA was used as reported by Nakai et al. (*3*). HSS included in the HSA software package was especially useful in this study because our intention was to investigate the pattern similarity of different segments within peptide sequences.

Homology Similarity Search. A preliminary study was conducted by changing the size of segments (three to six residues) to determine the most appropriate size of segment within the peptide sequence for rational elucidation of the emulsification mechanism. Using the segment size thus selected, the search was initiated from the N terminus by shifting the search one position at a time toward the C terminus. For every segment, the pattern similarity constant and the average of property index values were computed. The property indices used for amino acid side chains were hydrophobicity, charge, propensities of α-helix, β-strand, and β-turn, hydrogen bonding, and bulkiness as reported previously (*12*). In this study, the HSS was conducted for three-residue segments ELE using peptide S (1 in **Table 1**) with the highest EAI value as a reference. The segment ELE (positions 1−3) in peptide S was chosen rather than LEL (positions 2−4) for potentially better emulsification effects.

Instead of using gapped multiple-sequence alignment as in the previous paper (*3*), ungapped amino acid sequences were directly analyzed without gaps between amino acid residues. This choice was made because it was unlikely that evolutionary influences were involved in peptide sequences affecting their emulsifying property. Furthermore, to investigate the polar/apolar cycle, gaps will interfere with the true distribution computation. Partial molar compressibilities of amino acids (*14*) were added as a property index to the HSA program to investigate the effects of molecular flexibility of peptides on their emulsifying property, although its effect may not be as great as in the case of larger molecules such as proteins.

All peptides used for the functional sequence analysis in this study are shown in **Table 1**. The only peptides with available emulsifying ability data were included in this table. EAI data of peptides derived from α_{s1}- and β-caseins (*15*−*17*) may be comparable as they have been reported from the same laboratory as Saito et al.'s (*8*). Although other measures of emulsification, for example, oil droplet size, may be also useful for the purpose of this study, unfortunately, only limited data for a small number of peptides were available in the literature.

The HSA software including the HSS program used in this study along with instructions on how to use the computer programs is available as an ftp file (ftp://ftp.agsci.ubc.ca/foodsci/), downloadable to any PC.

RESULTS

Panels **A1**, **A2**, and **A3** of **Figure 1** are the HSS patterns of Saito's synthetic model peptides S, H, and R, respectively (*8*), drawn by using the hydrophobicity index of side chains. As shown in the pattern similarity curves of **Figure 1A1** (1 in **Table 1**) and **1A2** (2), a regularity in polar/apolar alternate distribution is required for good emulsification. The horizontal lines drawn above the similarity patterns show the location of high (solid line) and medium (broken line) hydrophobic similarity density (similarity/residue) estimated by visual comparison. Panels **A3** and **A4** of **Figure 1** show too broad intervals between the main similarity peaks in peptide R (3) and Sigma O-4632 (4). Panels **A7** and **A8** of **Figure 1** compare two CAP, that is, salmine (7) and bovine lactoferricin (8), respectively. Salmine suffers from irregular similarity compared to lactoferricin.

Figure 2 (**A9**−**A15**) illustrates the comparison of the fractions separated from hydrolysates of β-lactoglobulin and α_{s1}- and β-caseins (**Table 1**). Of the total of four fractions separated from β-lactoglobulin by Turgeon et al. (*18*), surface active fractions (f41−60) and (f21−40) in **Figure 2A10** (10) and **Figure 2A9** (9) and poorly surface active fractions (f61−70) and (f149−

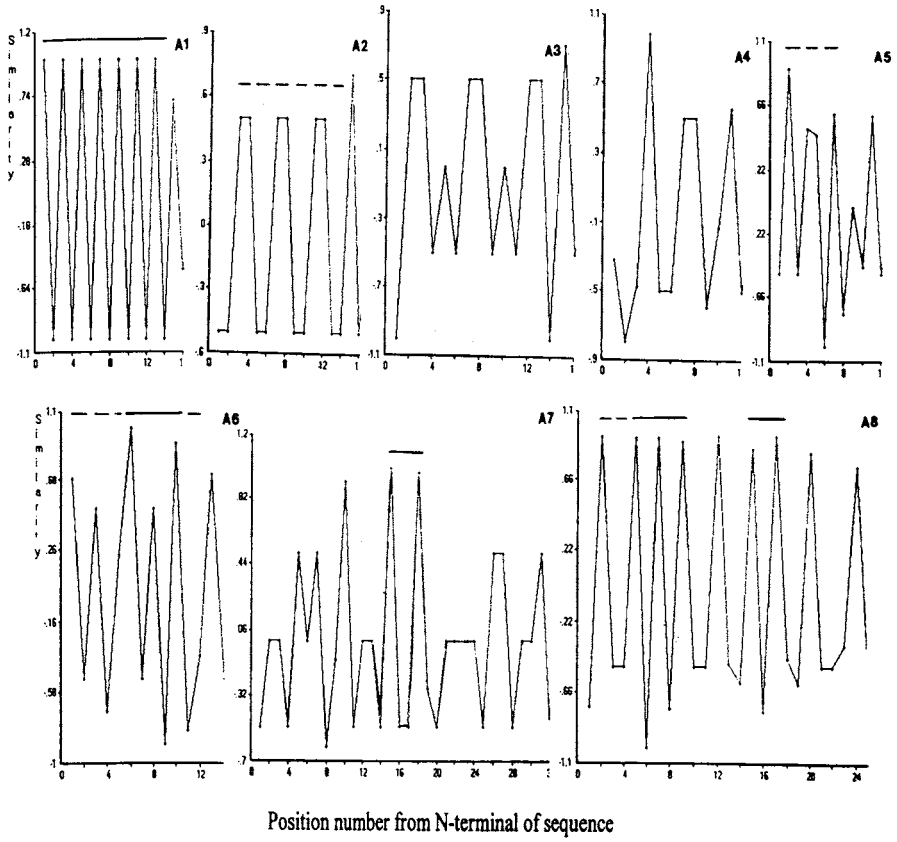

Figure 1. HSS patterns of peptides using hydrophobicity index I: A1, peptide S (1); A2, peptide H (2); A3, peptide R (3); A4, Sigma O-4632 (4); A5, Sigma F-9145 (5); A6, Sigma S-7152 (6); A7, salmine (7); A8, bovine lactoferricin (8).

162) in **Figure 2A11** (11) and **Figure 2A12** (12), respectively, are shown in this figure. It appears that hydrophobic similarity density is proportional to the absorption rate (AbsRate in **Table 1**) computed from surface tension measurement, which is closely related to emulsifying ability (*18*). **Figure 2A13** (13), **Figure 2A14** (14), and **Figure 2A15** (15) do not show strong periodicity, which may be reasonable to explain EAI values of 8−10 at high protein concentrations of 1−2%, which are expected to be lower in EAI at 0.1% protein (**Table 1**).

As shown in **Figure 3**, fraction β-Lg (149−162) (12) (**Figure 3B5**) possessed definitely lower regularity for compressibility than did fraction (41−60) (10) (**Figure 3B4**). However, no distinct difference in compressibility was observed between α_{s1}-CN (1−23) (13) and βCN (1−25) (14) (data not shown), although it was slightly better in the former, which would explain relatively low EAI values of 10 at 1.0 and 2.0%, respectively. Compressibilities of salmine and lactoferricin were not much different in the compressibility similarity (**B2** and **B3** of **Figure 3**), at least no high similarity. When helix and strand propensities were used in the HSS computation, there was no explicit trend to explain the emulsifying ability difference among all peptides analyzed in this study (**Figure 4**), except peptide S (**Figure 4C1**). This is especially true between Sigma peptide O-4632 (4) (**Figure 4C4**) and S-7152 (6) (**Figure 4C5**). This result appears to be contradictory to that of Saito et al., who reported the importance of helix structure in the emulsifying ability of their peptides (*8*).

Although bulkiness did not reveal difference in all peptides, charge distributions were not uniform in βCN (193−209) (15) (**Figure 6D8**), β-Lg (61−70) (11), and Sigma peptide O-4632 (4) (**Figure 5D4**), which was not greatly different from that of S-7152 (6) (**Figure 5D5**) without explaining the better emulsification of the latter with an EAI of 10 at 0.1%. Meanwhile, relatively high uniformity despite lower similarity constants was observed in salmine (**Figure 5D2**), which may explain the not extremely inferior EAI of this peptide with an EAI of 7 at 0.1% protein. **Figure 5D2** for salmine is unique, probably because of the positive charge of arginines providing some benefit to its emulsification capacity, which lactoferricin (**Figure 5D3**) does not have. **Figure 5D3** does not explain the relatively good EAI of 14 at 0.1% bovine lactoferricin.

Under acidic conditions, enhancement of the emulsifying ability was reported for peptide H (2) (*8*), α_{s1}CN (1−23) (13) (*15*), βCN (1−25) (14), βCN (193−209) (15) (*16*), and βLg (41−60) (10) (*18*). It appears that the presence of aspartic and glutamic residues with isoionic points of side chains at 3.9 and 4.3, respectively, may be a cause. At pH 4.0, both side chains dissociate about half, which would alter the polar/apolar balance because of the greater nonpolarity of the undissociated form of the carboxyl group. α_{s1}CN (1−23) (**Figure 6D7**) and βCN (1−25) (**Figure 6D9**) had some similarity in charge pattern and distribution to those of peptide H (**Figure 6D6**), which showed the highest EAI of the three peptides of Saito et al. (*8*) under acidic conditions: 43 for H versus 23 for S and 5 for R. However, this explanation is not acceptable for βCN (193−209) (15) shown in **Figure 6D8** without an acidic side chain within the sequence. The only possible explanation for good acidic emulsification by this peptide (15) is the inclusion of 4 proline residues in the sequence of 17 residues. We reported the characteristic behavior of a proline residue causing a molecular torsion (*19*). Because there are few available data on acidic emulsifying activity in the literature, confirmation of these hypotheses is unfortunately difficult.

Lipophilization of salmine was conducted using lauric, myristic, and palmitic chlorides. Assuming the binding sites of fatty acid chlorides to be amino groups or hydroxyl groups in

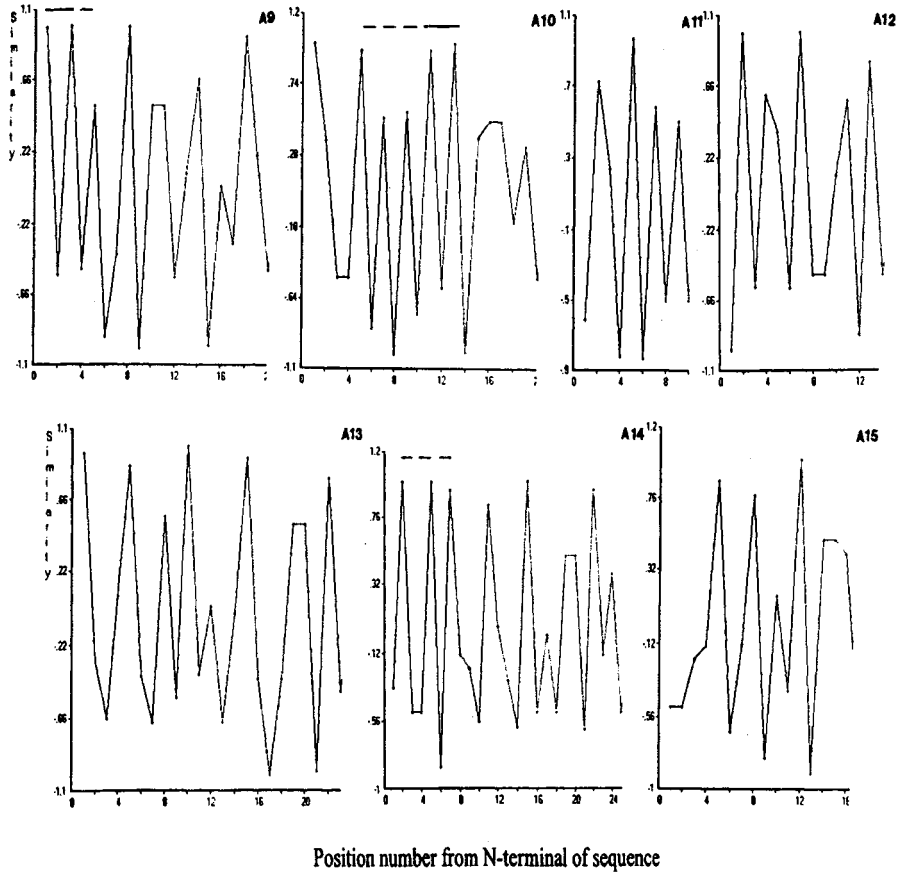

Figure 2. HSS patterns of peptides using hydrophobicity index II: A9, β-lactoglobulin (21–40) (9); A10, β-Lg (41–60) (10); A11, β-Lg (61–70) (11); A12, β-Lg (149–162) (12); A13, α_{s1}-casein (1–23) (13); A14, β-casein (1–25) (14); A15, β-casein (193–209) (15).

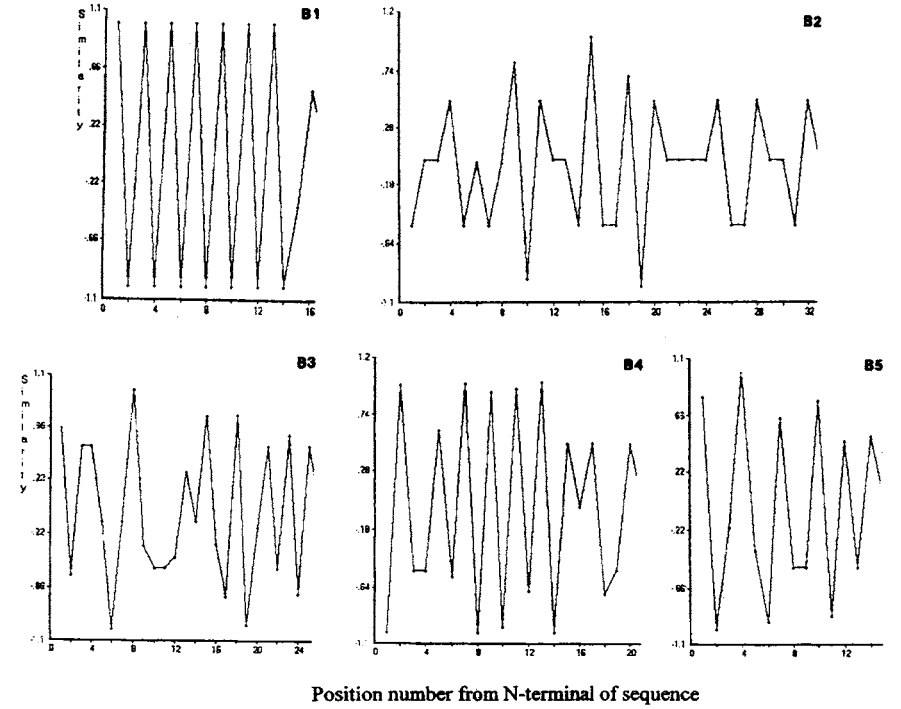

Figure 3. HSS patterns of peptides using compressibility index: B1, peptide S (1); B2, salmime (7); B3, lactoferricin (8); B4, β-lactoglobulin (41–60) (10); B5, β-lactoglobulin (149–162) (12).

salmine sequence, and log *P* values of fatty acids to be 5, 6, and 7 according to Rekker (*20*), these values were used as hydrophobicity values of hydrocarbon chains of the fatty acids, which had been added to the lipophilized sites. This modification in hydrophobicity computation was made on the basis of the fact that a close similarity was found between the hydrophobicity index used in this study and the values computed using Rekker's hydrophobic fragment constants (*20*). The HSS program was

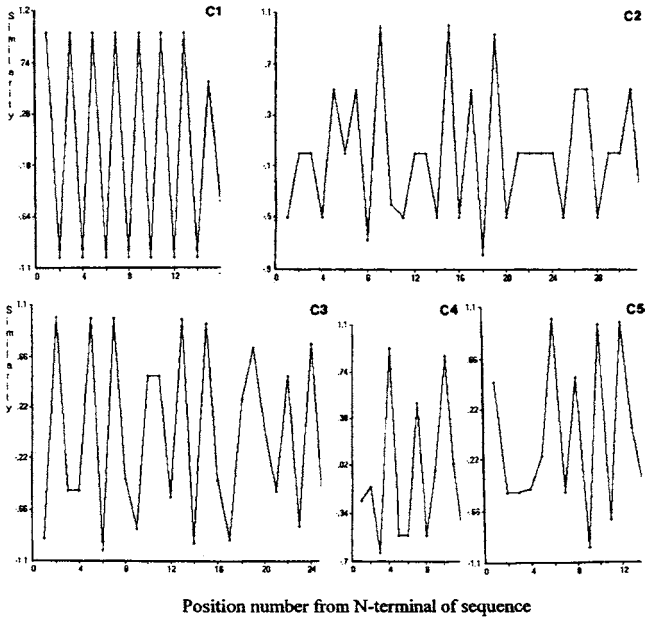

Figure 4. HSS patterns of peptides using helix propensity index: C1, peptide S (1); C2, salmine (7); C3, lactoferricin (8); C4, Sigma O-4632 (4); C5, Sigma S-7152 (6).

therefore modified to accommodate hypothetical binding positions and fatty acid (FA) selected and then followed by the HSS profile computation. The results of the computation are shown in **Figure 7** with a better periodicity pattern in **Figure 7E2** (double FA) than in **Figure 7E1** (single FA), and both are superior to the unlipophilized control (**Figure 1A7**). It appeared that selection of the binding position was more important than that of fatty acid. These results are supportive of the improved emulsification of salmine by lipophilization. Unfortunately, the binding sites could not been determined in this study.

Although direct comparison between the above peptides is difficult due to the lack of reliable data conducted under identical conditions, the good emulsifying ability of toxic peptide melittin with 26 residues (*21*) could be explained using the HSS program in a similar manner (data not shown as no comparable emulsification data are available). Melittin showed a pattern similar to that of lactoferricin (**Figure 1A8**) or β-Lg (21−40) (**Figure 2A9**) possessing fairly good periodicity. Melittin is strongly amphipathic and consequently is capable of interacting with biological membranes, leading to cell lysis (*21*). This property is also required for antimicrobial peptides including salmine and lactoferricin.

A crude correlation study of all available EAI data in **Table 1** was attempted. Hydrophobic periodicity was visually estimated on the basis of the hypothesis that highly periodic polar/apolar cycles alone are effective in oil emulsification of a sequence without counting irregular zones within the same sequence. The correlation thus yielded is illustrated in **Figure 8**. This finding, in addition to close relations of hydrophobic pattern similarity with emulsifying capacity of within-group peptides, such as three peptides of β-lactoglobulin (9−12) and Sigma peptides (4−6), would support the importance of hydrophobic distribution of peptide sequences in emulsification more than those of charge and compressibility. The HSS patterns of lipophilized salmine in **Figure 7** also may be supportive of our hypothesis for their enhanced emulsification, although the binding sites of the fatty acid radicals in the salmine sequence have not been identified.

DISCUSSION

The result shown in **Figure 8** demonstrates the usefulness of the three-residue motif ELE, which is the minimum unit for pattern similarity computation, to search for hydrophobic units within sequences. The zones including the periodic sequence of this motif with restricted distance (**Figure 1A1**,**A2**), but not

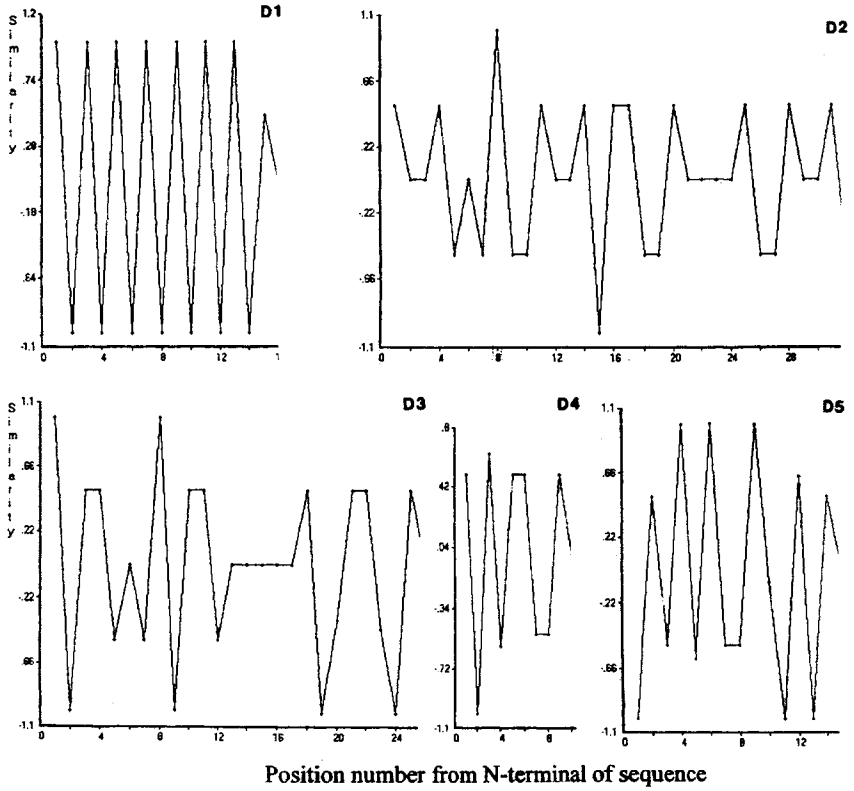

Figure 5. HSS patterns of peptides using charge index I: D1, peptide S (1); D2, salmine (7); D3, lactofericin (8); D4, Sigma O-4632 (4); D5, Sigma S-7152 (6).

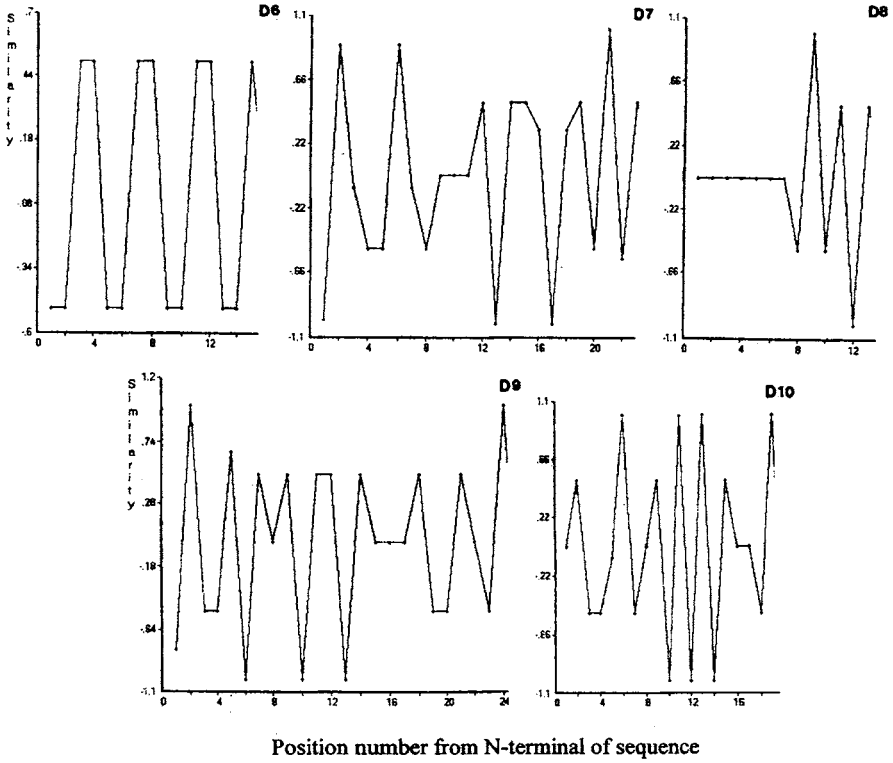

Figure 6. HSS patterns of peptides using charge index II: D6, peptide H (2); D7, α_{s1}CN (1–23) (13); D8, βCN (193–209) (15); D9, βCN (1–25) (14); D10, β-Lg (41–60) (10).

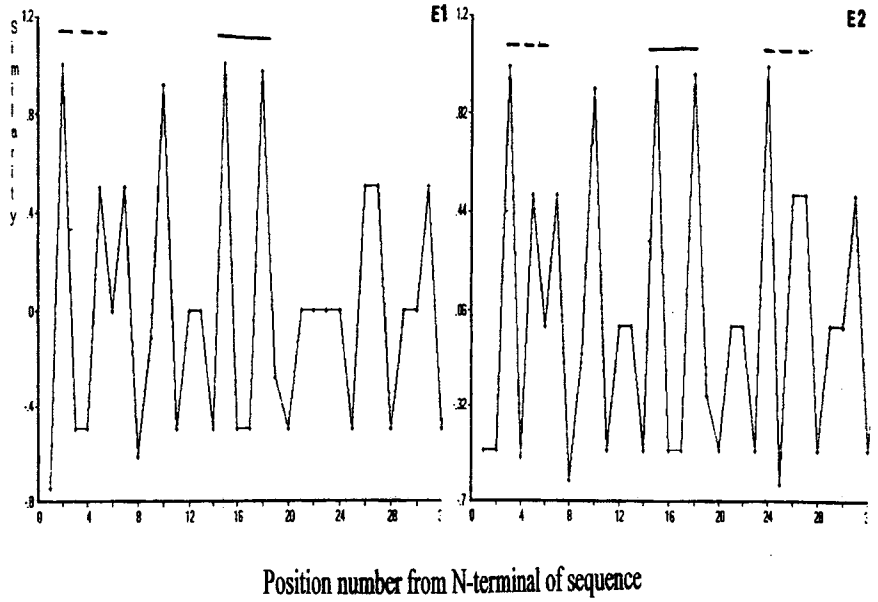

Figure 7. HSS patterns of lipophilized salmine using hydrophobicity index: E1, lauric acid bound at position 3; E2, palmitic acid and myristic acid bound at positions 4 and 25, respectively.

too far apart (**Figure 1A3**) are required for forming a good emulsion. Another typical example of this hypothesis is shown in **Figure 1A4**−**A6** and **Figure 2A9**−**A12**. Uniform periodicity of the hydrophobic similarity density may be required in peptides for their high emulsification activity. This concept is not the same as the hydrophobicity periodicity as discussed by Giuliani et al. (*11*). Also, the absorption rates shown in **Table 1** are comparable only within the same laboratory.

Turgeon et al. (*18*) stated that characteristics essential to good interfacial properties for peptides are clustering of hydrophilic and hydrophobic residues in distinct zones and minimum molecular mass allowing this distribution. They have corroborated that peptides with good interfacial properties are characterized by a distribution of hydrophobicity in discrete regions with three to five residues when separated by two or three polar residues. They also stated that uniform distribution of hydrophobic and hydrophilic amino acids may be required. Despite a general acceptability for explaining peptide emulsification, these hypotheses are not be fully applicable to the differences in emulsifying ability of Saito's peptides (*8*). In Saito's peptides, an alternate alignment of one hydrophobic residue after one hydrophilic residue showed the best emulsifying ability. The later hypothesis later proposed by Huang et al. (*10*) emphasized the importance of charge density and distribu-

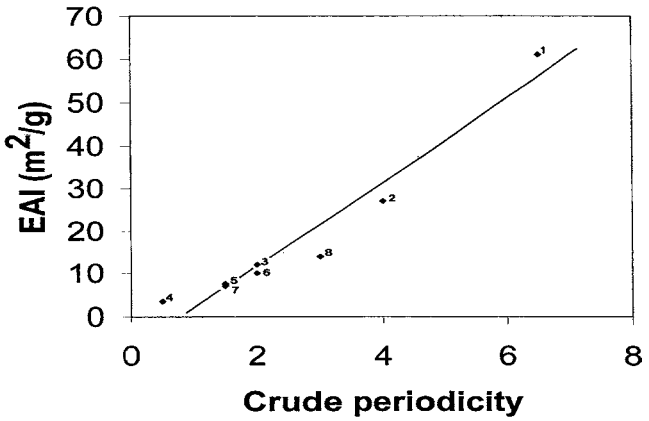

Figure 8. Correlation of EAI with visual estimate of periodicity of hydrophobicity distribution of peptides. The periodicity estimated here is the total of similarity constants within segments with a repeat of about the same similarity constants. The number label for each data point is the peptide number shown in **Table 1**.

tion. Although charge constitutes high polarity, effects of other radicals such as polyols (e.g., saccharides) in glycosylated proteins on hydrophilicity cannot be ignored (*22*).

The emulsifying ability of short-chain peptides may be elucidated from amino acid sequences using pattern similarity of hydrophobicity along with a supplemental role of structural flexibility (inverse compressibility), whereas charge effects may become important when emulsifying ability under acidic conditions is considered. In general, our results are in reasonably good agreement with those of Huang et al. (*10*). In the meantime, proline has a unique geometry by restricting the conformational freedom of the backbone of polypeptide chains (*23*). However, the effect of proline introduction into the active site of neutral protease was not straightforward. It was possible that the presence of a glycine residue at the N-terminal side of proline residue may greatly release strain caused by that specific residue, thereby resulting in structural stabilization (*19*).

An effective role of secondary structure could not be observed in this study, although its importance may not be ruled out when the size of the peptides increases as discussed in several papers (*8*, *21*, *24*). The importance of helix structure within sequences of CAP for invasion of microbial cell matrix was discussed in our lactoferricin paper (*3*). However, as far as emulsifying property alone is concerned, the role of the α-helix should not be much emphasized except that the α-helix has some hydrophobic periodicity.

An interesting discussion was made by Turgeon et al. (*18*). The improvement of emulsifying properties of α_{s1}CN (1−23) (13) in **Figure 2A13** by α_{s1}CN (f154−199) in mixture, which was observed by Shimizu et al. (*17*), can be explained by distinct hydrophobic zones separated by polar residues. Our HSS pattern of fraction α_{s1}CN (154−199) was similar to that of βCN (1−25) in **Figure 2A14** (14), which was better than that of fraction α_{s1}CN (1−23) in **Figure 2A13** (13) in terms of hydrophobic pattern similarity, but without much difference in the compressibility pattern similarity along with relatively high average compressibility values between α_{s1}CN (1−23) and (f154−199). This may have provided a better emulsifying ability to the latter.

Surface hydrophobicity of the entire molecule may be important in explaining the adsorption to the oil/water interface as observed by Shimizu et al. (*15*), but not necessarily closely correlating with emulsification (*17*). Furthermore, structural changes in proteins are widely observed during complex formation with ligands, substrates, inhibitors, and so forth (*25*). Therefore, surface hydrophobicity is difficult to define and may be variable under different circumstances. This phenomenon is compensated by our results on lactoferricin and its derivatives (*3*), which emphasized the importance of segments in the sequence for explaining peptide functionality. However, the application of the HSS program to explain peptide emulsification is not the same as HSA analysis for functional motifs in sequences to justify the difference in antimicrobial activity of peptides (*3*). In contrast to the homology analysis for the latter purpose, emulsification ability was investigated by applying the HSS approach to the ungapped sequences in this study. The results thus obtained are suggestive of the irrelevance of revolutionary conservation with peptide emulsification.

To experimentally identify functional motifs such as emulsification capacity within sequences, the most useful methods may be the measurement of emulsifying properties of fractions of known sequences separated from protein hydrolysates or of peptides chemically (*8*) or genetically synthesized in a much larger spectrum than the peptides employed in this study. It is generally agreed that site-directed mutagenesis is one of the most effective methods for structure−function study. It is quite likely that site-directed derivatization of peptides by using chemical synthesis may be the most powerful tool for structure−function study of small peptides in the future. After the collation of abundant reliable data of synthetic oligopeptides and their derivatives, it may be feasible to accurately predict emulsifying properties by conducting QSAR studies as was done for lactoferricin and its derivatives (*3*).

CONCLUSION

Pattern similarity between segments in HSS profiles of sample sequences using the motif ELE in the reference peptide with the best emulsifying capacity as a search unit is effective for determining the positions of periodicity as defined as hydrophobic similarity density in polar/apolar cycles within the sample sequences. This hydrophobic periodicity appears to play the most important role in peptide emulsification. Hydrophobicity distribution in peptide sequences is thus the crucial criterion for forming stable oil in water emulsions, although emulsion stability per se is not discussed herein as its mechanism is not exactly the same as of emulsifying properties.

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