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Quantitative Structure−Activity Relationship of Prolyl Oligopeptidase Inhibitory Peptides Derived from β-Casein Using Simple Amino Acid Descriptors

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Quantitative structure−activity relationship (QSAR) modeling using simple amino acid descriptors was done on a set of prolyl oligopeptidase (POP) inhibitory peptides derived from β-casein. Using partial least-squares regression, a QSAR model was obtained indicating that increased hydrophobicity and molecular bulkiness of amino acids in positions P3, P2, and P1′ of inhibitory sites on peptides resulted in increased inhibition (lower IC_{50}). Proline residues were always assumed to be in position P1. Hydrophobicity and molecular bulkiness have also in other studies been found as important factors for binding between substrates (or inhibitors) and the active site of POP. Prolyl oligopeptidase in blood serum is found to influence the level of hormone and neuropeptides and be related to cognitive and neurological deficiencies, e.g., Alzheimer's disease. Increased knowledge of the relationship between peptides derived from food proteins and their POP inhibition may be important in the development of functional foods as a supplement to pharmaceutical agents.

KEYWORDS: Milk; casein; prolyl oligopeptidase; PEP; POP

INTRODUCTION

Milk proteins are generally regarded as healthy because they are a good source of amino acids required for growth and maintenance of the body. However, recent research has pointed out that they may contain physiologically active (bioactive) peptides released through proteolysis (*1*). Health-related effects of bioactive milk peptides are related to the cardiovascular [e.g., angiotensin I-converting enzyme (ACE) inhibitory peptides and antithrombotic peptides], nervous (e.g., opioid peptides), immune (e.g., immunomodulating), host defense (e.g., antimicrobial peptides), and nutritional system (e.g., casein phosphopeptides) (*2*). They have potential as neutraceuticals in functional foods, a perspective that has been thoroughly reviewed (*3*).

Among the bioactive peptides, of which their health-related mechanisms are related to inhibition of physiological proteolytic enzymes, a major interest and research have been on ACE inhibition and its relevance to blood pressure and the cardiovascular system. However, bioactive peptides derived from food proteins may also interfere with other important physiological enzymes in the body. One of those enzymes that has been shown to be inhibited is prolyl oligopeptidase (POP). POP preferentially hydrolyzes peptide bonds on the carboxyl side of proline residues and has selectivity for oligopeptides. Because proline exists as an imino rather than an amino acid, most peptidases are not able to hydrolyze the peptide bond at proline residues. However, peptide hormones and neuropeptides usually contain one or more proline residues. Processing and degradation of such physiological peptides require proline-specific enzymes such as POP (*4*). The activity of POP in serum is therefore found to influence the level of hormone- and neuropeptides that further affect cognitive and neurological functions. The relationships between POP activity and cognitive or neurological deficiencies are further supported by clinical findings. Patients with different stages of depression, mania, schizophrenia, or Alzheimer's disease have been found with abnormal POP activity levels in blood serum. Pharmaceutical agents that affect POP activity have therefore been developed in the treatment of such deficiencies (*5*). The enzyme also has a role in the regulation of blood pressure by participating in the renin−angiotensin system through the metabolism of bradykinin and angiotensins I and II (*6*). Peptides inhibitory to POP in vitro have already been identified and characterized from β-caseins (*7*, *8*), sake (*9*), and wine (*10*). POP inhibitory peptides derived from food proteins may be a supplement to pharmaceutical agents and be the active ingredient in a functional food targeted toward patients with mental deficiencies in a similar way as has been done with foods containing ACE inhibitory peptides relative to cardiovascular diseases.

To further explore the possibility of POP inhibitory peptides as an active substance in functional foods, more specific information on relationships between peptide structure and inhibition activity is desired. An ideal would be to quantitatively relate peptide sequence to inhibitory activity. Such models may then be used for prediction and synthesis of active peptides as well as to provide a better understanding of the physicochemical mechanisms involved. In medical sciences and toxicology, quantitative structure−activity relationship (QSAR) modeling has been used extensively for such purposes. The idea behind

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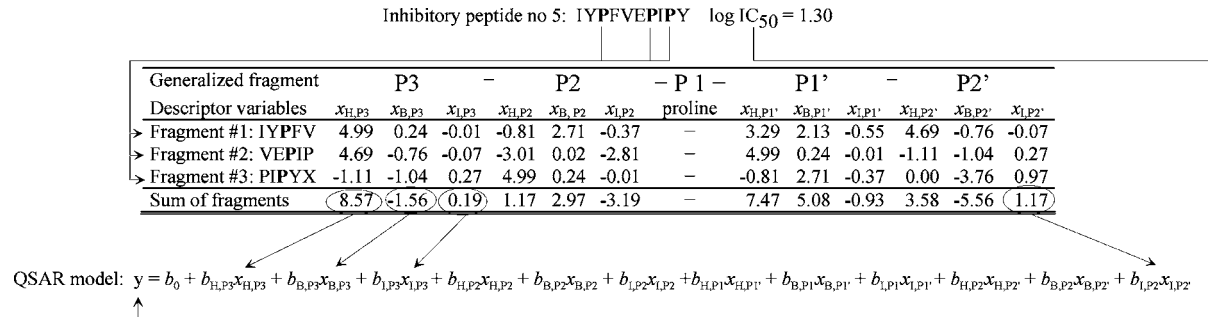

Figure 1. Example of descriptor treatment and calculations of a generalized inhibitory fragment from a POP inhibitory peptide. Proline residues in endoposition (bold letter) with adjacent amino acid residues were assumed to act as sites of competitive inhibition. The additive effect of several sites of competitive inhibition was expressed by summing up the descriptor values for the sites. The linear regression model was analyzed by partial least regression.

Table 1. Selected Peptides Derived from β-Casein and Their Inhibition of POP Expressed as IC_{50} (μM)[a]

no.	peptide	log IC_{50}	no.	peptide	log IC_{50}
1	IY**P**FVE**P**I	0.90	17	PVE**P**I**P**Y	1.74
2	LIY**P**FVE**P**I	0.95	18	IY**P**FVE	1.85
3	HL**P**L**P**L	1.00	19	IY**P**FVEP	1.85
4	IY**P**FVE**P**IP	1.08	20	IY**P**FV	2.00
5	IY**P**FVE**P**I**P**Y	1.30	21	VY**P**F**P**G**P**I	2.04
6	Y**P**FVE**P**I**P**Y	1.30	22	VY**P**F**P**G**P**IH	2.04
7	LY**P**FVE**P**I	1.30	23	IH**P**FAQTQ	2.18
8	PFVE**P**I**P**Y	1.40	24	VE**P**I**P**Y	2.26
9	IY**P**F**P**G**P**I	1.40	25	IY**P**	2.30
10	IF**P**FVE**P**I	1.40	26	Y**P**FVE**P**IP	2.40
11	PI**P**Y	1.48	27	IY**P**V**P**E**P**S	2.45
12	IY**P**F	1.56	28	IY**P**SFQ**P**Q	2.48
13	VY**P**F**P**G**P**IA	1.65	29	E**P**I**P**Y	2.56
14	VY**P**F**P**G**P**IP	1.70	30	Y**P**FVE**P**I	2.60
15	IY**P**F**P**G**P**IH	1.70	31	PFVE**P**I	2.88
16	IY**P**F**P**G**P**IP	1.70	32	FVE**P**I	2.99

[a] Data compiled from Asano et al. (*7*, *8*).

this methodology is that biological activity is a function of chemical structures that can be described by molecular or physicochemical variables, e.g., electronic attributes, hydrophobicity, and steric properties (*11*), resulting in multivariate data. Introduction of computer software and development within multivariate statistics and chemometrics including powerful multivariate regression methods such as partial least-squares (PLS) and artificial neural networks has enhanced the application of QSAR modeling. A subfield in QSAR research, referred to as peptide QSAR, has been established. This has been demonstrated as a useful approach in the areas of taste, physical properties, and ACE inhibition by peptides in foods (*12*) and has been used to evaluate food proteins as sources of bioactive peptides (*13*).

The present objective was thus to perform such modeling on POP inhibitory peptides derived from β-casein using simple amino acid descriptors that are related to well-known physicochemical properties.

MATERIALS AND METHODS

POP Inhibitory Peptides. The primary structure of POP inhibitory peptides derived from β-casein of different origin and their respective inhibition of activity expressed as peptide concentration (μM) required to inhibit enzyme activity by 50% (IC_{50}) was compiled from a study on synthetic peptide fragments (*7*, *8*). Peptides with $IC_{50} > 1000$ μM and peptide derivates were excluded from QSAR modeling (**Table 1**).

Amino Acid Descriptors. Physicochemical properties hydrophobicity, bulkiness of side chain, and charge of amino acids were expressed by the Kyte-Doolittle hydropathy scale (*14*), normalized van der Waals volume (*15*), and isoelectric point (*16*), respectively. However, the QSAR modeling approach used required that the absence of an amino acid in the sequence was described by physicochemical properties. For hydrophobicity, the average of Kyte-Doolittle hydrophaty scale, for bulkiness the normalized van der Waals volume similar to glycine, i.e., 0, and for charge an isoelectric point of 7.0 were used to describe the absence of an amino acid in a peptide fragment. Descriptor values were then standardized to an average value of zero before use in QSAR modeling (**Table 2**).

Table 2. Amino Acid Descriptors for Hydrophobicity (x_H), Molecular Bulkiness (x_B), and Isoelectric Charge (x_I) Using Kyte-Doolittle Hydrophaty Scale (*14*), Normalized van der Waals Volume (*15*), and Isoelectric Point (*16*), Respectively[a]

amino acid	code	x_H	x_B	x_I
Ala	A	2.29	–2.76	–0.03
Arg	R	–4.01	2.37	4.73
Asn	N	–3.01	–0.81	–0.62
Asp	D	–3.01	–0.98	–3.26
Cys	C	2.99	–1.33	–0.98
Glu	E	–3.01	0.02	–2.81
Gln	Q	–3.01	0.19	–0.38
Gly	G	0.09	–3.76	–0.06
His	H	–2.71	0.90	1.56
Ile	I	4.99	0.24	–0.01
Leu	L	4.29	0.24	–0.05
Lys	K	–3.41	1.01	3.71
Met	M	2.39	0.67	–0.29
Phe	F	3.29	2.13	–0.55
Pro	P	–1.11	–1.04	0.27
Ser	S	–0.31	–2.16	–0.35
Thr	T	–0.21	–1.16	–0.37
Trp	W	–0.41	4.32	–0.14
Tyr	Y	–0.81	2.71	–0.37
Val	V	4.69	–0.76	–0.07
none	X	0.00	–3.76	0.97

[a] The value expressing absence of amino acids (none) was derived (see text), and each descriptor was standardized to an average of zero.

Inhibitory Fragments for QSAR Modeling. The QSAR modeling approach and treatment of descriptor values are illustrated in **Figure 1**. It was assumed that POP inhibition was related to proline residues in the inhibitory peptides (**Table 1**). Only proline residues in endopositions were considered, with the exception of peptide no. 25 that had only a terminal proline residue. The proline residues were assumed to interact with the active site (position P1). Properties of residues adjacent to the proline in N- and C-terminal direction, i.e., positions P3, P2, P1′, and P2′ according to the Schechter and Berger nomenclature for proteolytic activity on peptides, were assumed to potentially influence the degree of inhibition. The descriptors for these four residues were used as variables in the modeling, giving a total of 12 variables in the QSAR model (**Figure 1**). Several peptides had more than one proline residue in endoposition that could be related to inhibition activity, and experimentally determined inhibition would therefore likely be due to the additive inhibitory effects of these sites. The physicochemical descriptors were therefore summed up for the assumed inhibitory

peptide fragments to obtain a physicochemical expression of the additive effect of inhibitory sites on the peptides.

Regression Analysis and Model Optimization and Validation. A data set of 32 samples with *y* response being POP inhibition expressed as log IC_{50} (μM) and a data matrix **X** with 12 variables expressing physicochemical properties of additive inhibitory peptide fragments were generated. PLS regression analysis was conducted to estimate a QSAR model. The variables in **X** were centered and standardized to equal variance before regression analysis. A method for assessing the uncertainty of parameters in multivariate regression models developed by Martens and Martens (*17*) and integrated into the software package was used to reduce and optimize the regression model. Regarding regression methods (*18*), the multiple correlation coefficient (R) is considered to represent the proportion of the variance explained by the model (model fit). The cross-validated R (designated Q) represents the ability of a model to make predictions with new data (model predictive ability). Regression analysis was performed with the Unscrambler software, version 9.1 (Camo Process AS, Oslo, Norway). The regression model was validated by full cross-validation.

RESULTS AND DISCUSSION

QSAR models express the biological activity of compounds as a function of structural variation. Those structural variations have then to be expressed by descriptors. For peptide QSAR, descriptors for the individual amino acids are often used and especially if the activity is strongly related to the primary structure. Such descriptors can be common physicochemical quantities for amino acids or descriptors derived especially for use in peptide QSAR (*12*). Hydrophobicity, molecular bulkiness, and charge are typical properties that can influence biological activities of peptides and have also been used in peptide QSAR modeling of ACE inhibitory activity (*19*). The modeling approach chosen (**Figure 1**) required that also absence of amino acids in positions adjacent to the prolyl residue was expressed by descriptors. The average of Kyte-Doolittle hydrophaty scale was chosen to avoid a strong effect in such cases. Because absence of an amino acid will consequently give a low degree of molecular bulkiness, the lowest value for bulkiness of an amino acid (i.e., glycine) was chosen. An isoelectric point corresponding to neutral pH was chosen to express the low effect of charge in such conditions.

Improved biochemical insight into binding mechansims of oligopeptides to the active site of POP has mainly been obtained by inhibitory studies and the binding of peptides to inactivated POP. On the basis of those studies, it is assumed that POP binds to adjacent residues to the prolyl group in the peptide and that those residues play a role in the catalytic cleavage at the carboxy side of prolyl residues in peptides (*4*, *20*). It was for QSAR modeling assumed that the peptides derived from β-casein (**Table 1**) act through competitive inhibition and that several prolyl residues with adjacent residues can form inhibitory sites. PLS regression gave using all 12 variables in regression analysis a model with $R = 0.85$ and after full cross-validation $Q = 0.70$. None of the samples were regarded as outliers. The regression model was reduced using the method by Martens and Martens (*17*), and equivalent predictability was found by the model:

$$y = 2.69 - 0.038x_{H,P3} - 0.16x_{B,P3} - 0.13x_{H,P2} - 0.18x_{B,P2} - 0.091x_{H,P1'} - 0.089x_{B,P1'} \quad (1)$$

where the *x*-variables express hydrophobicity and molecular bulkiness in positions P3, P2, and P1′ and *y* is POP inhibition expressed as log IC_{50}. The other variables in the original QSAR model (**Figure 1**) were not found to significantly improve predictability. This reduced model gave a predictability of $R = 0.84$ and with full cross-validation $Q = 0.73$ (**Figure 2**). The

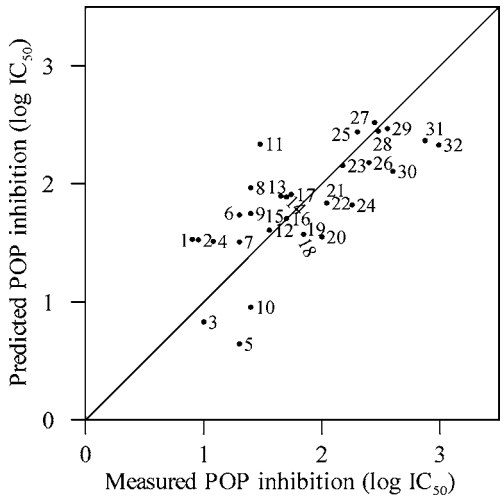

Figure 2. Predicted vs measured values for inhibition of POP by peptides derived from β-casein using the QSAR model (eq 1) indicating that hydrophobic and bulky amino acids adjacent to proline residues give inhibitory peptides ($R = 0.84$, $p < 0.001$). The plot is based on values obtained after full cross-validation ($Q = 0.73$, $p < 0.001$). Numbers correspond to peptides in **Table 1**.

QSAR model in eq 1 expressed that increased hydrophobicity and molecular bulkiness of amino acids in positions P3, P2, and P1′ give an increased degree of POP inhibition (lower IC_{50}). Earlier studies have indicated the importance of hydrophobicity of the side chain residues (*21*−*24*).

To our knowledge, peptide QSAR modeling has not previously been done on food protein-derived peptides inhibitory to POP, but structure−activity relationship investigations have been reported for other synthesized POP inhibitors or modified peptides revealing that large hydrophobic residues at the N-terminal of inhibitors greatly improved their potency (*21*, *22*, *25*). Binding mechanism studies of the active site in POP have also shown that hydrophobic residues attached to the side chain of the amino acid in P2-position increase the affinity between enzyme and substrate (or inhibitor) (*26*). Protein structural studies by Fülöp et al. (*27*) have shown that the S3 subsite in the active site of POP provides a rather nonpolar environment and is further lined by the side chains of several nonpolar residues, including Phe173, Met235, Cys255, Ile591, and Ala594. Consequently, this subsite prefers interaction with hydrophobic residues. Examination of the active site gave that Cys255 was situated close to the S1 and S3 subsites. This was further given as a major reason that inhibitor studies with a bulky reagent specific to cysteine residues inactiviated POP almost completely, whereas a smaller inhibitor gave only partly inhibition. On the basis of the biochemical information about POP structure and the interaction between substrates or inhibitors with active site, a proposed interpretation of the results obtained by the QSAR modeling (eq 1; **Figure 2**) would be that hydrophobic amino acids in positions P3, P2, and P1′ give a strong interaction between peptide and active site, but bulky amino acids in the same peptide positions inactivate POP giving an effective inhibition of the enzyme. Thus, a peptide with hydrophobic and bulky amino acids would both bind efficiently at the active site and inhibit the enzyme from cleaving the peptide and thereby becoming an inhibitory peptide to POP.

The assumption in this QSAR study was that the inhibitory efficiency of the peptides derived from β-casein was mainly decided by their interaction with the active site of POP. Structural studies of POP have shown that it is made up of a peptidase unit and a seven-bladed β-propeller that covers the

active site and excludes larger peptides and proteins from the active site (*27*). A previously proposed mechanism implies that the oscillating propeller blades act as a gating filter allowing only shorter peptide substrates into the active site (*28*). However, recent structural studies have shown that the propeller domain is fairly stable and rigid (*29*). This finding has been further supported by molecular dynamic simulation, which identified a small tunnel at the interdomain region comprising the highly flexible N-terminal segment of the peptidase domain and a facing hydrophilic loop from the propeller as the only potential pathway for the substrate toward the active site (*30*). The domain interface is thus found to have a critical role in the interdomain dynamics and substrate specificity of POP. A rather complicated dynamic mechanism where the incoming peptide substrate induces a conformational change of the enzyme causes the domain interface to open and where extent of the substrate−catalytic−domain interactions depends on the size of the peptide has been proposed (*31*, *32*). The limited surface area created by the domain opening would, thus, efficiently select the size of the oligopeptides and protect larger structured peptides and proteins from proteolysis. Possible dynamic effects or blocking of the pathway on POP by the inhibitory peptides is not taken into account in this reported QSAR study. As more research will be available on the dynamic catalytic mechanism of POP, it may be possible using so-called three-dimensional QSAR techniques and molecular simulations to better explore such effects.

An improved understanding of the structure−activity relationship of POP inhibitory peptides would be helpful in the search for food proteins and proteolytic treatments that could make a substantial contribution to functional food ingredients aimed at individuals with certain neurological and cognitive deficiencies. Research into the stability of peptides during gastrointestinal degradation and on the bioavailability of peptides are areas that must be examined further if such functional foods should be developed.

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