J. Kor. Pharm. Sci., Vol. 25, No. 3, S37-S42 (1995)

Peptide Drug Delivery: a Method to Overcome Enzymatic Barrier

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(Received April 21, 1995)

Recently, many attempts have been made to develop a convenient and reliable method for non-parenteral absorption route of peptide and protein therapeutics. However, peptides and proteins tend to be rapidly degraded by proteases even at the site of administration. This enzymatic barrier to proteinaceous drug absorption is believed to be largely responsible for incomplete bioavailability of these substances. Therefore, the need exists or rational strategies to minimize systemic absorption of proteinaceous drugs.

Some approaches that aim at reducing proteolysis include (a) structural modification of peptide backbone, N-and C-termini, and labile amino acid residue and (b) co-administration of protease inhibitors. Nevertheless, such approaches require a basic knowledge of the nature of enzymatic barrier. This includes understanding of types, magnitude, and regional distribution of proteolytic enzymes in the mucosal membranes and how these will influence systemic bioavailability of a given peptide or protein. In the present study, many of these aspects of the enzymatic barrier have been examined using substance P as a model peptide and the eye as an alternate route to parental administration. Based on the obtained information, additional experiments were performed to assess the effectiveness of using protease inhibitors and structural modification of substance P in protecting the model peptide from the peptide-degrading enzyme system.

Materials and Methods

Preparation of Tissue Homogenates and Subcelluar Fraction

Tissue collection—Tears were collected from a group of ten female albino rabbits, weighing 2.5~3kg. After collection, the tears were centrifuged at 1,000×g for 10 min to remove contaminating lipids. In the meantime, ocular tissues (i.e., the conjunctiva, corneal epithelium, cornea stroma, sclera, iris-ciliary body and lens) were excised and aqueous humor was withdrawn from albino rabbits immediately after their decapitation.

Preparation of homogenates—Tissues were homogenized in a Potter-Elvejhem homogenizer. A 5.0 mM-histidine/imidazole buffer, pH 7.4, containing 5% w/v sorbitol, 0.5 mM Na_2EDTA[1)] was used (buffer : tissue wet weight = 10 :1, v/w). The homogenates were centrifuged at 1,000×g for 10 min (4℃). The homogenates were as a source of crude enzyme for initial studies.

Preparation of subcellular fractions—For the isolation of the membrane and cytosolic factions, the So was centrifuged at 38,000 rpm for 40 min (Beckman L5-65 Ultracentrifuge). The resulting supernatant (S1) was retained, whereas the pellet (P1) was used as crude membrane preparation. The membrane fraction (P1) was further subfractionated by equilibrium density-gradient sedimentation according to a modified procedure of

Mircheff et al.[1)]

Characterization of subcellular fractions—Marker enzymes for subcellular fractions included alkaline phosphatase (brush border), Na^{+}-K^{+}-ATPase (basolateral membrane), N-acetyl glucosamidase (lysosome), NADPH-cytochrome c reductase (endoplasmic reticulum) and succinate dehydrogenase (mitochondria) based upon their cellular compartmentalization.[2)] Utilizing well-establised biochemical assays for each marker, an assignment can be made to the possible subcellular origin of that population.

Hydrolysis of peptides

Assay—Substance P and its syntheic analogs were incubated, in duplicate, with tissue homogenates in phophate-buffered saline (PBS, pH7.4) at 37℃ for various intervals of time up to 3h. The enzymatic reaction was terminated by the addition of acetonitrile. After centrifugation, the supernatant was transferred to a microvial and the acetonitrile was evaporated. Samples were reconstituted with mobile phase prior to HPLC assay. In each assay one or more of the following controls were included in duplicate: zero-time control, zero-enzyme controls and incubated enzyme controls (tissue extract placed at 37℃ for 3h). The reaction products were resolved by HPLC equipped with an Ultrasphere ODS column(75 mm× 4.6 mm, 5 m; Beckman). The mobile phase was a mixture of acetonitrile and 0.1 M sodium perchlorate containing 0.1% phosphoric acid, pH 2.1. The peptides were eluted as single peaks at a flow rate of 1 m*l*/min using a step gradient system. All the peptides eluted were detected and quantified by monitoring the absorbance of the eluate at 210 nm.

Amino acid analysis—Samples were hydrolyzed with 6 N-HC*l* at 100℃ for 14 h in sealed and evacuated tubes which were made from glass test-tubes. The constituent amino acids in free form yielded were labeled with 1-fluoro-2,4-dinitrobenzene (FDNB) with reacts with the α-amino group of a free amino acid to yield a 2,4-dinitrophenylamino acid. Duplicate runs were made on HPLC C-18 column.

Effect of inhibitors—The effect of various inhibitors on the different enzyme activities was tested (Table III). The tissue homogenates were preincubated with the inhibitors for 10 min at 37 ℃. Controls in which the tissue extracts were preincubated in the absence of inhibitors were included. ICB and retinal subcelluar fractions were studied for substance P-hydrolyzing activities by repeating substance P incubation under conditions where specific protelysis was prevented by the inclusion of selected proteinase inhibitors.

Data analysis—The open one-or multi-compartment kinetic models were adopted to describe the kinetics of peptide degradation. An estimate of the formation rate comstant (k_f) for each metabolite compound was obtained by fitting an appropriate model to the data using the statistical computer program PCNONLIN(Statisical consultants, INC., Lexington, Kentucky). Each substance P fragment was fitted to more than one model and the equation with minimun Akaike's Information Criterion (AIC)[3)] was regarded as the best representation of the time course plot.

In general, the appropriate equation of describe the time course of phenylalanine and methionine was a sequential (A-B-C) metabolite formation model,

$$C=C_{max}[1-k_{f2}\ e^{-k_{f1}}\ t/k_{f2}-k_{f1})-k_{f1}\ e^{-k_{f2}}\ t/(k_{f1}-k_{f2})] \quad (1)$$

where C is the concentration of a given species at time t, C_{max} is the maximun concentration attainable, k_{f1} is a first order rate constant for the formation of the intermediate, and k_{f2} is a first order rate constant for the formation of the amino acid from the intermediates. The rising portion

of the concentration-time profile for SP9-11 in the corneal stroma, lens and tears was also fitted to this model.

On the other hand, the rising protion of the concentration-time profiles for both and C-terminal fragments except Gly-Leu-Met-NH_2 tripeptide (SP9-11)in the corneal stroma, lens and tears, were best fitted to a product accumulation model,

$$C=C_{max}\ (1-e^{-k}f) \quad (2)$$

where k_f is a first order rate constant of product formation. The declining portion (i.e.,after the time when the formation apparently had ceased) of the concentration-time profiles for most of fragments except SP1-2, phenylalanine and methionine was described by a monoexponential curve,

$$C=Cpe^{-ket} \quad (3)$$

where Cp is concentration of SP5-11 at the breakpoint, and ke is a first order elimination rate constant. The break point most likely corresponds to the time when the overall hydrolytic activities on substance P and its fragments was about to exceed the activity to release a given fragment.

Preliminary nonliear regression analysis of the data obtained for several final metabolites indicated that failure to include a lag time resulted in systematic bias. This bias could be eliminated by inclusion of a lag time estimated from each specific data set in equations (1) and (2) for several final metabolites of phenylalanine, methionine and SP-2 in most of tissues as well as for SP9-11 in the tears, corneal stroma and lens. The apparent lag time was estimated graphically by back extrapolation of the initial rising phase of the concentration-time curve to zero concentration.

Results and Discussion

The principal objective of the research described in this study was to examine the nature of the enzymatic barrier in the ocular peptide delivery in order to design strategies for peptide drug delivry. To accomplish this objective, we first examined the distribution of peptide-hydrolyzing activity in the anterior segment of ocular tissues and tears in vitro by using a model peptide substance P (Arg-Pro-Lys-Gln-Gln-Phe-Phe-Gly-Lue-Met-NH2). Substance P hydrolyzing activities were detected in all the ocular tissues examined. Substance P was rapidly hydrolyzed at several sites witihin its backbone resulting in a complex fragmentation pattern as shown in Figure 1. The levels of activity showed over 30-fold regional differences as listed in Table 1;the activities in the conjunctiva and sclera were the highest, whereas those in the tears and lens were the lowest. Moreover, an important outcome of this study was the observation that distribution of aminopeptidase activity closely matched the substance-P hydrolyzing activity. This indicates that aminopeptidase may be a major peptidase involved in substance P degradation, in spite of the unique strctural feature of the peptide with penultimate proline residues at N-terminus.

By quantitatively comparing the fate of hydrolytic products of substance P, it was found that ocular peptidases involved in the major attack on

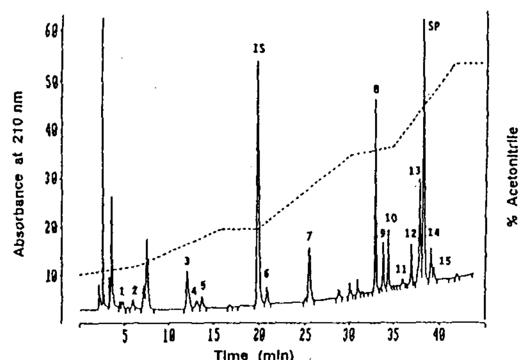

Figure 1—A typical HPLC chromatogram of substance P and its hydrolytic products.

Table I—Degradation Rate Constants for Substance P Incubated with Tears and Homogenates of Anterior Segment Tissues from the Albino Rabbit Eye

Tissue	Degradation rate constant k (min^{-1}/ 50 μg protein)×100
Tears	0.17 ± 0.01
Conjunctiva	5.21 ± 0.35
Corneal epithelium	2.57 ± 0.12
Corneal stroma	1.40 ± 0.07
Sclera	4.11 ± 0.34
Iris-cilliary body	2.24 ± 0.09
Lens	0.29 ± 0.02

The values represent means ± SD of three experiments. Data obtained for the queous humor is not shown due to the low protein con tent in the aqueous humor preparation.

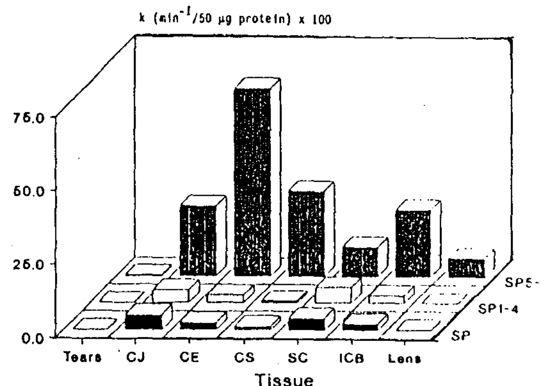

Figure 2—First order rate constants for the hydrolysis of substance P (SP), SP_{5-11} and SP_{1-4} incubated with tears and homogenates of anterior segment tissues from albino rabbit eye

substance P exhibited that the relative proportion of the various proteases varies from tissue to tissue as reflected by different pathways of substance P fragmentation among ocular tissues. The strongest substance P-degrading primary activity, reflected by the values of formation rate constant and maximum attainable concentration for the model peptide fragments, can be attributed to Pro[2]-Lys[3] and Gln[6]-Phe[7] bonds cleaving activity in the conjunctiva and sclera, Pro[4]-Gln[5] and Gln[6] -Phe[7] bonds cleaving activity in tears, corneal epithelium, corneal stroma and iris-ciliary body, and Pro[2]-Lys[3], Pro[4]-Gin[5] and Phe[7]-Phe[5] bonds cleaving activity in the lens.

This tissue variation in substance P-degrading activitiy indicates regional differences in the type and magnitude of constituent enzymes in the peptide-hydrolyzing system. This is suggested by the hydrolysis of two partial sequences of substance P (SP1-4 and SP5-11) which showed that the tissue variation in enzymatic activities was more directed towards the C-terminal sequences than the N-terminal sequences(Figure 2).

By elucidating the cleavage sites of substance P based on the known specificity of peptidases and fragments formed, a general two-step mechanism of substance P degradation in the ocular tissues is suggested. First, the primary attack on substance P occurred by the action of several endopeptidases which include post-proline cleaving enzyme (PPCE), endopeptidase-24.11 (ENKase) and angiotensin-converting enzyme(ACE). The only exopeptidase involved in the primary attack on substance P appeared to be dipeptidyl aminopeptidase IV (DAP IV). Second, exopeptidases further degraded most of the primary metabolites (i.e., secondary attack). In particular, the direct action of aminopeptidases was so efficient that it facilitate the overall degradation of substance P.

To determine the subcellular distribution of ocular peptidases involved in the substance P degradation, rabbit retinal subcellular organelles were isolated by differential centrifugation in sorbitol density gadient. Based on the kinetic examination and marker enzyme enrichment (Table II), it can be concluded that (1) substance P-hydrolyzing peptidases are present differentially in soluble and membrane-bound forms;PPCE appears to be in greater abundance in the cytosolic fraction, whereas ENKase, ACE and DAP IV are relatively enriched in the membrance fraction, (2) each fraction is capable of substance P inactivation

Table II—Initial Rate Constants for the Formation of Substance P Fragments in Retinal Homogenate and Cytosolic (SI) and Membrane (P1) Fractions

Fragment	Activity ko (nmol/min)×100			Ratio
	So	S1	P1	S1/P1
1-4	12.29 ± 0.51	12.24 ± 1.81	0.88 ± 0.10	13.9
1-7	7.94 ± 0.52	7.40 ± 0.10	1.39 ± 0.14	5.3
1-9	4.32 ± 0.30	1.41 ± 0.40	33.61 ± 2.61	0.04
1-11 (FA)	1.44 ± 0.05	0.46 ± 0.07	6.81 ± 1.01	0.07
3-11	2.81 ± 0.46	2.76 ± 0.04	0.37 ± 0.16	7.5
5-11	9.49 ± 0.69	8.12 ± 1.79	2.12 ± 0.39	3.8
6-11	34.51 ± 4.12	22.73 ± 2.83	12.15 ± 1.26	1.9
8-11	10.45 ± 0.76	9.66 ± 0.44	0.86 ± 0.06	11.2
9-11	6.20 ± 0.09	5.72 ± 0.28	0.85 ± 0.09	6.7

Values represent means ± SD obtained from three experiments.

in a concerted mechanism of primary cleavage of the peptide by endopeptidases and secondary action on substance P intermediate metabolites by exopeptidases and (3) soluble aminopeptidase degrades C-terminal fragments rapidly, thereby influnencing the overall rate of fragmentation in the soluble enzyme preparation.

The extent to which selective peptidase inhibitor(s) protect the model peptide was estimated in retinal and ICB homogenates. In these experiments, several inhibitors were found to be effective as listed in Table III. First, the inhibition with aminopeptidase inhibitor(amastatin) showed that the indirect action of aminopeptidase contribute over 25% of total substance P-hydrolyzing activities. Second, the use of phosphoramidon and captopril verified the involvement of ENKase and ACE, respectively. Third, When thiol reagents (PCMPS, PCMB) were used as inhibitors, the hydrolysis of substance P was inhibited by over 80% of control. These compounds were the most effective inhibitors use in stabilzing substance P aganist proteolysis.

Additional experiments using analogs of substance P were performed to assess the effectiveness of structural modification of substance P in protecting the peptide from the substance P-degrading enzyme system. Among synthetic analogs of substance P that were designed to resist metabolic degradation,[4] a topically effective substance P antagonist, [D-Pro[2], D-Trp[7,9]] SP (SP-ptt), and a heptapeptide agonist, [pGln,[5] MePhe,[8] MeGly[9]] SP 5-11(DiMe-C7), were chosen in the present study based on the findings obtained above. The sites of protection against enzymatic cleavage by introducing chemically modified amino acid or D-amino acid in the original sequenecs of substance P and SP5-11. The susce-

Table III—Effects of Peptidase Inhibitors on Substance P (0.25 mM) Metabolism upon Incubation for 15 and 30 min at 37℃ with the Homogenate of Iris-ciliary Body (100 mg protein/ml)

Inhibitor	Inhibitor concentration	% of Control 15 min	% of Control 30 min
None (control)		100	100
EDTA	1 mM	88*	92
1,10-Phenanthroline	1 mM	88*	92
Dithiothreitol	1 mM	76**	84*
Phosphoramidon	1 mM	93	96
Thiorphan	50 μM	92*	94
Captopril	0.5 μM	80*	89
Iodoacetic acid	2 μM	153***	107
P-Chloromercuribenzoate	0.05 mM	82**	ND
	0.5 mM	21***	29***
P-Chloromercuriphenylsulfonate	1 mM	4***	18***
Diisopropylfluorophosphate	1 mM	60***	76**
Phenylmethylsulfonyl fluoride	1 mM	60**	76**
Phenylmethylsulfonyl fluoride	1 mM	82*	84*
Aprotinin	1 mg/ml	87*	89
Amastatin	0.1 mM	91**	97**
Bestatin	1 mM	64**	79**
L-Prolylprolinal	2	93	95
Pepstatin A	50	99	100
Leuptin	0.1 mM	89*	97

Resuits are expressed as a percentage of the amount of substance phydrolyzed in a control incubation in which no inhibitor was added. Each value is the mean of three experiments (s.d.<20%). In the control, 52.9% and 31.5% of the substance P remained intact at 15 and 30 min, respectively. N.D., not determined. Asterisks indicate stastically significant differences from control value obtained in paired t-test. *0.05>P>0.01; **0.01>P>0.001; ***P>0.001

Table IV—Summary of Rate Constants for the Hydrolysis of Substance P Analogs in Homogenates (50 Protein) of Ocular Tissues of the Albino Rabbit

Tissue	SP-ptt		DiMe-C7	
	Degradation rate constant		Degradation rate constant	
	(min^{-1}, 10^3)	SP-ptt/SP*	(min^{-1},10^3)	DiMe-C7/SP_{5-11}*
Tears	0.04 ± 0.01	0.024	0.18 ± 0.02	0.040
Conjunctiva	6.31 ± 0.89	0.12	6.20 ± 0.59	0.026
Corneal epithelium	3.16 ± 0.42	0.12	2.33 ± 0.70	0.0036
Corneal sstroma	0.18 ± 0.10	0.013	0.08 ± 0.14	0.0033
Sclera	12.04 ± 5.80	0.29	12.74 ± 1.33	0.13
Iris/Ciliary Body	5.22 ± 0.73	0.23	15.21 ± 0.64	0.066
Lens	0.02 ± 0.01	0.0069	0.11 ± 0.01	0.0017

The values represent the mean ± SD of tree experiments.
*rate constants for the hydrolysis of parent compounds (substance P and SP_{5-11}) are listed in Table 10.

ptibilities of both analogs to proteolytic action in ocular tissue homogenates were different from tissue to tissue as listed in Table IV. Upon incubation with various ocular tissue homogenates, SP-ptt was more resistant to enzymatic degradation than substance P, by factors of 42.5 in the tears, 77.8 in the corneal stroma, and 145 in the lens. The replacement of Pro[2] with D-Pro and both Phe[7] and Gly[9] with D-Trp reduced the rate of inactivation most significantly in those tissues with weak substance P-hydrolyzing activity. Similarly, a heptapeptide DiMe-C7 was more stable than SP5-11 in all the tissues examined. The stabilization factor of DiMe-C7 as compared with SP5-11 was 25.0 in the tears, 38.9 in the conjunctiva, 275.0 in the corneal epithlium, 298.8 in the corneal stroma, and 598.2 in the lens. On the other hand, the stabilization factor resulted from the modifications in positions 5,8 or 9 of SP5-11 as compared with substance P was 9.4 in the tears, 8.4 in the conjunctiva, 11.0 in the corneal epithelium, 14.3 in the corneal stroma, 3.2 in the sclera, 1.5 in the ICB and 26.4 in the lens.

Conclusion

The bioavailability and biological activity of peptides when used as ocular drug is governed by the enzymatic as well as other barrier. Any approach aimed at circumventing this barrier must be directed towars understanding the type, distribution, and properties of peptidases in a given peptide. Using substance P in ocular tissue preparations, I have established a model system to characterize the enzymatic barrier. As shown in the present study, the experimental system developed in the present study can be used to design and evaluate approaches aimed at reducing proteolysis of peptide drugs.

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