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APPLICATION OF THE MULTIPIN PEPTIDE SYNTHESIS TECHNIQUE FOR PEPTIDE RECEPTOR BINDING STUDIES: SUBSTANCE P AS A MODEL SYSTEM

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Abstract: *The multipin peptide synthesis technique allows thousands of discrete peptides to be synthesised simultaneously. To explore the application of this technique in large scale screening of bioactive peptides, substance P and analogues were synthesised and their receptor binding affinity determined. The results are consistent with published data, suggesting that this approach is applicable to large scale, quantitative receptor studies.*

The discovery of endogenous peptides that act as neurotransmitters or hormones has revealed new approaches for drug intervention in a variety of diseases. However, peptides have a high degree of structural diversity. This presents a challenge to any comprehensive, systematic study, as the capacity of conventional peptide synthesis is limited by the cost and the work load involved. Several techniques emerged in the 1980's to increase the capacity of peptide synthesis through parallel processing[1,2]. Among them the multipin technique has certain advantages[2]. By assembling peptides on polyethylene pins arranged in a microtitre plate format, parallel handling and subsequent testing of large numbers of peptides becomes viable. The technique has been used with success to identify antibody epitopes[2-4] and T-cell determinants[5-6] through large scale systematic screening. This highly systematic approach is potentially useful for research on bioactive peptides in many other biological fields.

A recent development in the technique now allows a very large number of support-bound peptides to be cleaved simultaneously as C-terminal amide products[7]. These peptides can be reconstituted into physiological buffers and used immediately for bioassays. To explore the high-volume applications of this technique for quantitative assays of bioactive peptides, a pilot study has been conducted with substance P (SP), an important peptidergic neurotransmitter/neuromodulator in the central nervous system as well as in many peripheral organs[8]. Structurally, SP belongs to a family of tachykinin peptides. At least three tachykinin receptor subtypes have been identified[9]. Since the discovery of SP in 1970, the structure-function relationships of the peptide have been extensively studied[8-13]. This information provides a useful benchmark for our study.

Table 1. Sequences of SP and its Analogues

Substance P	(SP)	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Neurokinin A	(NKA)	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neurokinin B	(NKB)	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
Physalaemin	(PHY)	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Eledoisin	(ELE)	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Kassinin	(KAS)	Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2

In the present study, the 6 mammalian and non-mammalian tachykinins listed in Table 1 were prepared. The peptides were synthesised by the multipin technique as described elsewhere[7], together with the 512 SP analogues presented in the following paper[14]. An Fmoc synthesis protocol was used, with couplings effected by PyBOP[15]. Following assembly and side-chain deprotection, the pin-bound peptides were subjected to a thorough washing protocol[16]. The peptides were then cleaved with ammonia vapour[7], which in this instance was provided by 25% ammonia(aq.). The resulting amide products were eluted from their respective pins into the wells of a microtitre plate with water/ethanol (1:1, 150 μL) and dried down. For reconstitution, the peptides were dissolved in 10 μL of 0.1 M acetic acid and diluted with 140 μL of receptor binding buffer[17]. Each pin gave, on average, 20 nmol of peptide. Amino acid analysis and HPLC indicated that the peptides were of *ca.* 85% purity. A positive ion FAB mass spectrum of SP (Fig. 1) showed that the product was partially oxidized at Met, with signals being observed at *m/z* 1348 ($[M+H]^+$) and *m/z* 1364($[M+O+H]^+$).

The receptor binding assay was performed as follows. An aliquot of rat brain synaptosomal preparation[10] (50 μg protein) was incubated with [^{125}I]Bolton-Hunter reagent labelled SP (BHSP) in receptor binding buffer[17]. The binding mixture (300 μL) was dispensed into 96 well microtitre plates. After incubation, the receptor-ligand complex was collected with a multiple channel cell harvester. The residual radioactivity was counted with a MultiGamma counter. Experiments were performed in duplicate and were repeated at least three times. Nonspecific binding of [^{125}I]BHSP was determined to be 10% of the total binding by competition with 10 μM unlabelled SP. The data obtained were analysed using a four-parameter logistic equation to determine the IC_{50} values and pseudo-Hill coefficients.

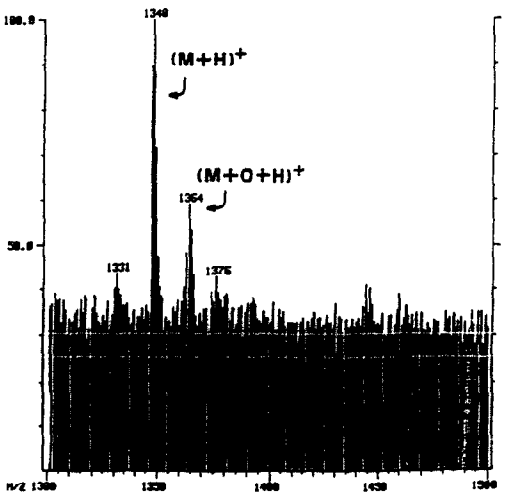

Fig. 1. FAB mass spectrum of SP

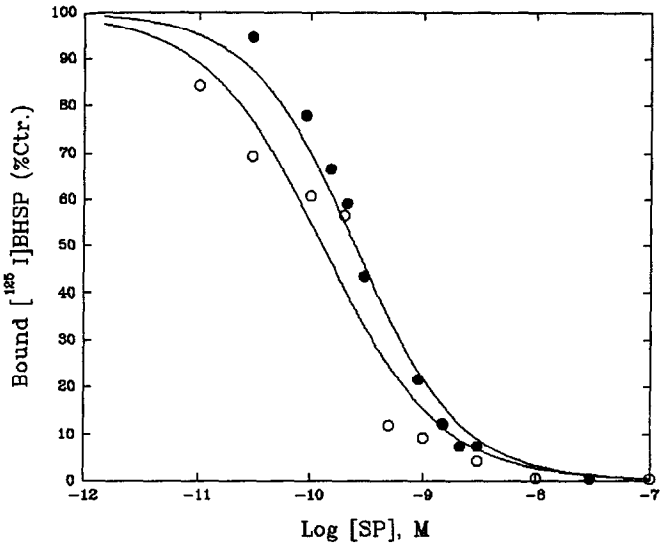

Fig. 2. Inhibition curves of SP synthesised by the multipin technique (•) and that purchased from a commercial source (Sigma) (o). Data are means of 2-3 determinations.

The inhibition curves of SP synthesised by the multipin technique and that purchased from a commercial source are shown in Fig. 2. The IC_{50} values of these two SP samples are very similar, being 0.37 nM and 0.16 nM respectively. The IC_{50} values of the 6 tachykinins tested in the present study are summarised in Table 2 along with the results reported in three recent studies[11-13]. These studies were performed in the rodent central nervous system tissue using [^{125}I]BHSP as radioligand. Our results are, in general, consistent with the reported data. The only exception is NKB, which showed a large variation throughout the literature. NKB binds preferably to the NK3 receptors[9,10] present at low levels in the brain with a different regional distribution to NK1 receptors[10,12]. Such variation could reflect a difference in the receptor preparations used for these studies.

Table 2. IC_{50} Values of Tachykinins: Comparison of the Present Results with Previous Data

Peptide	IC_{50} (nM)			
	present result	Ref.11	Ref.12	Ref.13
SP	0.37	0.38	0.30	0.77
NKA	32.0	49.0	38.1	66.0
NKB	870.0	100.0	57.3	444.0
PHY	2.30	-	4.20	0.53
ELE	31.0	17.0	61.7	69.0
KAS	75.0	-	84.8	-

There is a considerable variation in the primary structures and a broad range of binding affinities (from sub-nanomolar to micromolar) among the peptide analogues studied. Thus, the general agreement between the present study and the previous studies, where bulk synthesised and purified peptides were used, indicates that peptides synthesised with the multipin technique are suitable for a quantitative estimation of activity of potential drug candidates. This technique could find applications both in basic peptide research, and in large scale screening[14].

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17. The receptor binding buffer used was 50 mM Tris-HCl buffer (pH 7.5) containing 0.2% bovine serum albumin, 3 mM $MnCl_2$, 40 μg/mL bacitracin, 4 μg/mL leupeptin, 20 μg/mL soybean trypsin inhibitor type I-S, and 0.1 mM phenylmethylsulfonyl fluoride.