ASPERLICIN, A NOVEL NON-PEPTIDAL CHOLECYSTOKININ ANTAGONIST FROM *ASPERGILLUS ALLIACEUS*

FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

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The fermentation and isolation of a new, non-peptide cholecystokinin antagonist, asperlicin, produced by *Aspergillus alliaceus* is described. The potent and specific interaction of asperlicin with cholecystokinin receptors was shown using *in vitro* biochemical assays.

Cholecystokinin (CCK) is generally recognized as a classical gastro-intestinal transmitter involved in the control of pancreatic and gastric secretion, contraction of the gall bladder and gut motility. However, in spite of the broad interest in CCK, the few previously reported non-peptide antagonists of this neurotransmitter exhibit *in vitro* potencies only in the millimolar range. The present report describes the fermentation, isolation, and biological properties of a new, potent non-peptide CCK receptor antagonist from *Aspergillus alliaceus*. The compound designated as asperlicin is active in the micromolar range.

Fermentation

Material and Methods

Cultures: The *Aspergillus* species initially found to produce asperlicin was obtained from Dr. S. HERNANDEZ of Merck's microbial screening laboratory in Madrid, Spain. It and a natural isolate have been deposited at the American Type Culture Collection (ATCC) with accession numbers ATCC 20655 and ATCC 20656. *A. alliaceus* strain ATCC 16891 was obtained from the ATCC for this study. Another *A. alliaceus* strain NRRL 315 was obtained from the Northern Regional Research Laboratories of the United States Department of Agriculture. A Merck source of an *A. alliaceus* strain descended from NRRL 317 was also used in this study. All other *Aspergillus* strains used for this study were obtained from S. CURRIE of the Merck Sharp and Dohme Culture Collection.

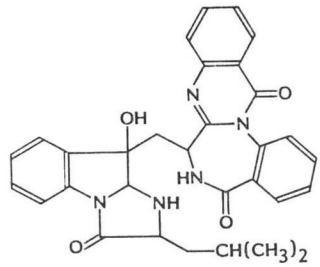
Fermentation Conditions: Inocula was obtained by growing the culture in a 250-ml Erlenmeyer flask containing 50 ml of medium I (Table 1). Inoculum flasks were agitated on a rotary shaker (220 rpm) for 2 days at 28°C and then its contents were used as vegetative inocula (5% inoculum). For a micro inoculum of spores and mycelia, flasks were incubated further statically at room temperature for 7~21 days. Spores and mycelia were then diluted with an equal volume of medium II (Table 1). Flasks were then agitated to disperse and wet the spores. Aliquots of this mixture, that

Table 1. Media composition.

Ingredient	Medium I (g/liter)	Medium II (g/liter)	Medium III (g/liter)
Corn-meal		10	
Corn-steep liquor	5		
Dextrose	10		40
Oat flour	10		
Tomato paste	40	30	
Trace element mix*	10 ml		
Yeast autolysate		10	
$CaCl_2 \cdot 2H_2O$	10		
$FeSO_4 \cdot 7H_2O$			0.01
KCl			0.5
K_2HPO_4			1
$MgSO_4 \cdot 7H_2O$			0.5
$NaNO_3$			5
$ZnSO_4 \cdot 7H_2O$			0.01
Distilled water	1,000 ml	1,000 ml	1,000 ml
Final presterile pH (NaOH - HCl)	6.8	5.0	7.0

* $FeSO_4 \cdot 7H_2O$ 1 g, $MnSO_4 \cdot 4H_2O$ 1 g, $CuCl_2 \cdot 2H_2O$ 0.025 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, H_3BO_3 0.56 g, $(NH_4)_6MoO_2 \cdot 4H_2O$ 0.019 g, $ZnSO_4 \cdot 7H_2O$ 0.2 g, distilled deionized water 1,000 ml.

Fig. 1. Structure of asperlicin.



would result in a 5% inoculum, were used to inoculate production media. Production media, at 40-ml per unbaffled Erlenmeyer flask, after inoculation, were incubated on a rotary shaker (220 rpm) for 5~7 days at 28°C.

Results

Fifty-eight strains of 24 genera of *Aspergillus* were tested for their ability to produce asperlicin. We have found significant levels of production from *A. alliaceus* (NRRL 315), *A. alliaceus* (descended from NRRL 317), *A. alliaceus* (ATCC 16891) and from our deposited cultures ATCC 20655 and ATCC 20656. ATCC 20655 has been identified as *Aspergillus alliaceus* Thom and Church by K. B. RAPER (personal communication). We have not produced significant levels of asperlicin from *Aspergillus* strains other than those identified as *A. alliaceus*.

Production of asperlicin occurs in a number of fermentation media and under a variety of fermentation conditions. In medium II (Table 1) titers of 15~30 μg/ml are produced by ATCC 20655 and ATCC 20656 after 6 days of incubation. The synthetic medium III (Table 1) supported production of 5~10 μg/ml after 6 days of incubation. Supplementing medium III with amino acids (2 g/liter) resulted in titer increases over the control (Table 2). Notably, supplementation with tryptophan

Table 2. Effect of amino acid supplementation on production of asperlicin.

Amino acid*	Titer** (μg/ml)	Amino acid*	Titer** (μg/ml)
L-Alanine	12	L-Isoleucine	18
D,L-α-Alanine	16	L-Leucine	12
L-Arginine	26	L-Lysine·HCl	16
L-Aspartic acid	15	L-Methionine	16
L-Asparagine	19	L-Phenylalanine	27
L-Cysteine	12	L-Proline	10
L-Cystine	7	L-Serine	19
L-Glutamine	18	L-Threonine	18
Glycine	23	L-Tryptophan	35
L-Histidine·HCl	11	D,L-Tryptophan	42
Hydroxyproline	18	L-Valine	15

* 2.0 g/liter supplementation of medium III, control titer 5 μg/ml.

** Titer determined by HPLC.

resulted in titers of 35~42 μg/ml. Tryptophan is a possible precursor of asperlicin the structure[1] of which is shown in Fig. 1.

Isolation and Purification

The fermentation broth (16 liters) was filtered through a bed of filter-aid. The mycelial cake was extracted twice with vigorous stirring with successive 3-liter portions of ethyl acetate; the broth filtrate was extracted once with 5 liters of ethyl acetate. The combined extracts were concentrated under reduced pressure to an oil which was triturated with 200 ml of ice-cooled petroleum ether; the precipitate that formed was collected by filtration and washed with a fresh 100 ml portion of cold petroleum ether. The solid was stirred with 500 ml methanol, the resulting suspension was filtered and the methanol removed under reduced pressure.

The resultant solid mass was taken up in 30 ml of methylene chloride - methanol, 9: 1, and applied onto a 250 g silica (E. Merck silica gel 60) column equilibrated with methylene chloride - methanol, 95: 5; elution was carried out with the same solvent mixture. The fractions containing the crude asperlicin (volume of retention *ca.* 2.2 column volumes) were pooled and evaporated to dryness under reduced pressure. The residue was dissolved in 10 ml of methylene chloride - methanol, 1: 3, and further fractionated on a column of Sephadex LH-20 (Pharmacia Fine Chemicals, column dimensions; 2.5×200 cm). Asperlicin eluted with a retention volume of 0.95 column volumes. The appropriate fractions were pooled and evaporated to dryness. Final purification of the compound was achieved by reverse phase liquid chromatography on a 1.4×183 cm column packed with E. Merck LiChroprep RP-18 25~40 μm particles using acetonitrile - water, 35: 65. Yield of pure asperlicin; approximately 85%. Its homogeneity was ascertained in several thin-layer chromatography systems (E. Merck Silica gel 60 F_{254}: methylene chloride - methanol, 95: 5, Rf 0.60; hexane - acetone, 2: 1, and ethyl acetate. Whatman $KC_{18}F$: methanol - water, 75: 25, Rf 0.70; and acetonitrile - water, 2: 1) and by HPLC (Whatman ODS-3, 10 μm particles, acetonitrile - water, 40: 60, 2.0 ml/minute, 40°C, $K'=5.3$).

In a larger scale isolation, whole broth (642 liters) was treated batchwise with 64 liters of Amberlite XAD-2 resin (Rohm and Haas Co.). After stirring for one hour, the suspension was filtered through a bed of filter aid. The mycelial resin cake was extracted twice in succession with 340 and 226 liters of acetone, stirring vigorously for 2 hours at ambient temperature. After filtration, the extracts were combined and concentrated under reduced pressure to an oil.

The oil was adsorbed onto 360 g of silica gel by stirring; after washing the silica gel with one liter of petroleum ether on a filtering funnel, the adsorbent was poured carefully onto the top of a silica gel chromatography column packed with 4 kg of silica gel 230~400 mesh. The column was washed with 20 liters of methylene chloride which removed most of the non-polar impurities. The compound of interest was eluted from the column with methylene chloride - methanol, 4: 1. The appropriate fractions were pooled and concentrated down to an oil in preparation for the next purification step. Further fractionation was achieved by passing the concentrate through another silica gel column (14 liters, packed in and equilibrated with methylene chloride - methanol, 95: 5). Elution with the same solvent mixture afforded asperlicin after 2.0 column volumes. Final purification was accomplished by multiple recrystallizations in methanol. Yield: 82%.

Physico-chemical Properties

Asperlicin was obtained as white crystals, melting at 211~213°C. It is soluble in methylene

Fig. 2. Asperlicin displacement of specific [^{125}I]-CCK-33 binding in rat pancreatic membranes.

Each point represents the mean±standard error of triplicate determinations replicated three times.

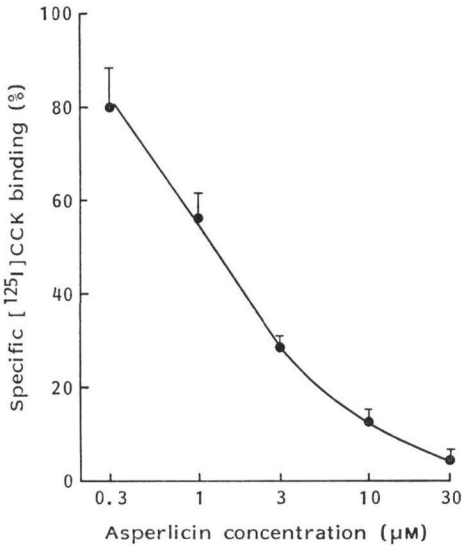
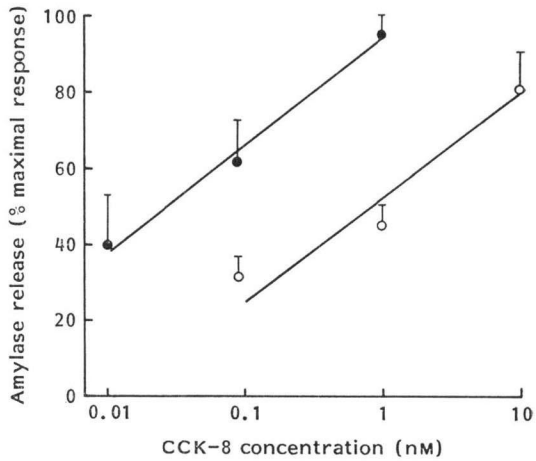


Fig. 3. Asperlicin antagonism of CCK-8 stimulated amylase release from rat pancreatic tissue.

The EC_{50} and 95% confidence limits for control (●) and asperlicin 13 μM (○) treated preparations were 2.5 (1.2~5.3)$\times 10^{-11}$ M and 8.1 (2.8~32.9)$\times 10^{-10}$ M respectively.

Each point represents the mean±standard error of four determinations.



chloride, acetone and the lower alcohols but virtually insoluble in water. Its UV spectrum exhibited the following maxima: λ_{max}^{MeOH} nm (ε) 322 (sh, 3,135), 310.5 (4,075), 278 (sh, 10,350), 266 (sh, 14,420), 258 (sh, 17,760), 230 (sh, 48,490). Optical rotation: $[\alpha]_D^{26.5}$ −185.3° (*c* 1.10, MeOH).

Biological Properties

The methods employed to study displacement of specific binding of [^{125}I]CCK from CCK receptors and CCK stimulation of amylase release in rat pancreatic tissue have been described previously[2,3].

Asperlicin displaced specific [^{125}I]CCK pancreatic receptor binding in a concentration-related manner (Fig. 2). The concentration causing half-maximal inhibition of binding (IC_{50}) was 1.4 μM. The specificity of asperlicin for CCK receptors was shown by its inability to significantly displace the specific binding of [^{3}H]diazepam, [^{3}H]naloxone, [^{3}H]angiotensin or [^{3}H]quinuclidinyl benzilate at concentrations as great as 20 μM.

Asperlicin (13 μM), alone, did not stimulate amylase release indicating a lack of agonist activity. However, asperlicin caused a 32-fold parallel shift to the right of the dose response curve for CCK-stimulated amylase release from pancreatic tissue (Fig. 3). The maximal response to CCK-8 in the presence of asperlicin did not differ significantly from CCK-8 alone, indicative of competitive antagonism. The dissociation constant (K_b) for asperlicin as an antagonist of CCK in this system was 0.4 μM.

The data demonstrate that asperlicin is a potent and selective antagonist of peripheral CCK receptors.

Acknowledgment

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