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History of yield improvements in the production of asperlicin by *Aspergillus alliaceus*

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SUMMARY

The natural product asperlicin is the first nonpeptide antagonist of cholecystokinin isolated from a microbial source. At discovery, production of asperlicin by the original soil isolate of *Aspergillus alliaceus* was between 15 and 30 mg/l. Selection of natural variants of *A. alliaceus*, use of Plackett & Burman and Simplex experimental designs; formulation of synthetic media; amino acid supplementation of production media; analysis of complex nitrogen sources for their amino acid content; evaluation of promising media in fermentors; substitution of glycerol for glucose as a carbon source and rational mutant selection all contributed to titer increases to >900 mg/l.

INTRODUCTION

The discovery of a potent nonpeptide cholecystokinin antagonist produced in a fermentation broth by a fungus *Aspergillus alliaceus* was greeted with much enthusiasm by our natural products scientists [5,7,10]. In this product, named asperlicin, we had the most potent nonpeptide cholecystokinin inhibitor known. Since that original observation minor broth constituents have been characterized [8,11]. By directed biosynthesis novel analogs have been prepared [9]. The biological activity of the asperlicins has been evaluated [5,12,13]. A widespread chemical effort was undertaken which has resulted in even more potent, more specific water-soluble antagonists [1,3,6]. Much of the above work relies upon the production of large quantities of asperlicin and upon the availability of high-titer broths. The studies undertaken to rapidly provide high-titer fermentations are the subject of this report.

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MATERIALS AND METHODS

A. alliaceus

The original culture found to produce asperlicin was obtained from soil isolated by Dr. S. Hernandez as part of a natural product screening program run in Madrid, Spain. This culture has been deposited in the American Type Culture Collection under accession number ATCC 20655.

Natural subisolates of ATCC 20655 were found to produce higher titers of asperlicin. They have been deposited in the American Type Culture Collection under accession numbers ATCC 20656 and ATCC 20738.

Seed media

Medium KF: tomato paste 60.0 g/l, corn meal 20.0 g/l, Ardamine pH 10.0 g/l, Polyglycol P2000 1.0 ml/l, presterile pH not adjusted.

Medium Syn 1: glycerol 12.5 g/l, NH_4Cl 1.0 g/l, KCl 0.5 g/l, K_2HPO_4 1.0 g/l , $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $FeSO_4 \cdot 7H_2O$ 0.1 g/l, $ZnSO_4 \cdot H_2O$ 0.1 g/l, Polyglycol P2000 1.0 ml/l, presterile pH 6.5.

Production media

Medium LG: tomato paste 30.0 g/l, corn meal 10.0 g/l, Ardamine pH 5.0 g/l, Polyglycol P2000 0.2 ml/l, presterile pH 5.0.

Medium 2X LG: tomato paste 60.0 g/l, corn meal 20.0 g/l, Ardamine pH 10.0 g/l, Polyglycol P2000 1.0 ml/l, presterile pH 5.0.

Medium PBS: Ardamine pH 5.0. g/l, $(NH_4)_2SO_4$ 2.0 g/l, corn meal 10.0 g/l, lard water 5.0 g/l, $CoCl_2 \cdot 6H_2O$ 0.01 g/l, soybean meal 5.0 g/l, KH_2PO_4 2.0 g/l, cod liver oil 2.0 ml/l, presterile pH not adjusted.

Medium PBG: dextrose 10.0 g/l, Ardamine pH 5.0 g/l, $(NH_4)_2SO_4$ 2.0 g/l, Polyglycol P2000 2.0 ml/l, glycerol 10.0 ml/l, soybean meal 5.0 g/l, tomato paste 5.0 g/l, sodium citrate 2.0 g/l, presterile pH not adjusted.

Medium X9: Medium S8 (Table 3) plus yeast RNA 1.0 g/l, *p*-aminobenzoic acid 0.1 g/l, anthranilic acid 0.1 g/l and L-tryptophan 1.0 g/l.

Medium III: dextrose 40 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, KCl 0.5 g/l, K_2HPO_4 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $NaNO_3$ 5.0 g/l, $ZnSO_4 \cdot 7H_2O$ 0.01 g/l, presterile pH 7.0.

Medium JK1: Ardamine pH 5.0 g/l, KH_2PO_4 2.0 g/l, $(NH_4)_2SO_4$ 1.0 g/l, corn meal 5.0 g/l, lard water 5.0 g/l, Pharmamedia 20.0 g/l, sodium citrate 2.0 g/l, L-phenylalanine 2.0 g/l, L-tryptophan 2.0 g/l, Polyglycol P2000 1.0 ml/l, presterile pH not adjusted.

Medium Syn B: L-phenylalanine 2.0 g/l, L-glutamate 3.0 g/l, NH_4Cl 1.0 g/l, KH_2PO_4 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, KCl 0.5 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, $ZnSO_4 \cdot 7H_2O$ 0.01 g/l, presterile pH 6.5.

Medium Syn 2: medium Syn 1 plus L-tryptophan 2.0 g/l, L-phenylalanine 2.0 g/l, monosodium glutamate $\cdot H_2O$ 5.0 g/l, presterile pH 6.5.

Medium EB33: glycerol 20.0 g/l, NH_4Cl 2.5 g/l, KH_2PO_4 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, KCl 0.5 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, $ZnSO_4 \cdot H_2O$ 0.01 g/l, $CoCl_2 \cdot 6H_2O$ 0.001 g/l, $CaCO_3$ 10.0 g/l, presterile pH 5.0.

Medium EBS: dextrose 40.0 g/l, $NaNO_3$ 2.5 g/l, KH_2PO_4 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, KCl 0.5 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, $ZnSO_4 \cdot 7H_2O$ 0.01 g/l, $CoCl_2 \cdot 6H_2O$ 0.001 g/l, $CaCO_3$ 10.0 g/l, presterile pH 5.0 before addition of $CaCO_3$.

Medium AL2 PM: glycerol 20.0 g/l, peptonized milk 37.5 g/l, Ardamine pH 1.0 g/l, presterile pH 7.0.

Culture conditions

All cultures were incubated with agitation at 28°C.

Analytical

After establishing linearity of detector response for injections of asperlicin ranging from 0.5 to 10 mg, the following protocol was used: Samples of fermentation broth were mechanically shaken with ethyl acetate for 4 min. After centrifugation a portion of the organic phase was taken to dryness. Dried residue was redissolved in methanol (1/5 to 1/2 original volume). Ten microliters of this solution was used for analysis by HPLC. A Whatman ODS-3 column, 10 μ (4.6 mm × 250 mm) was run at 40°C, eluted with CH_3CN/H_2O (40:60) at a flow rate of 2.0 ml/min. Detection was at 254 nm. Asper-

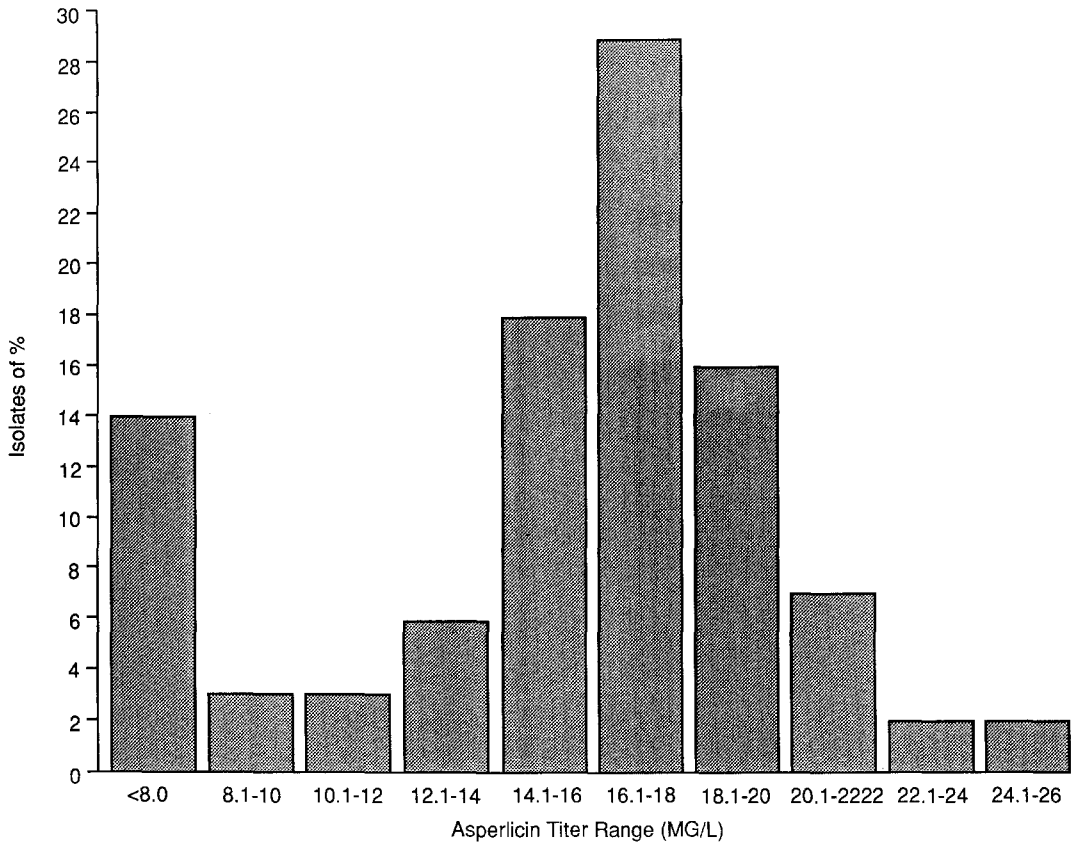

Fig. 1. Distribution of asperlicin production by natural isolates of *A. alliaceus*.

licin retention time was 6.9 min. With higher-titer fermentations it was possible to use samples prepared by the addition of an equal volume of methanol to the broth. After mechanical agitation for 5 min, followed by centrifugation, a 10 μl aliquot of the liquid portion was used directly for chromatographic analysis as above.

RESULTS

Microbial isolates from nature often are found to be a heterogeneous population of clones capable of producing markedly different titers of a secondary metabolite. *A. alliaceus* ATCC 20655 as isolated from nature showed an expected population distribution in its ability to produce asperlicin in LG production medium. The population distribution

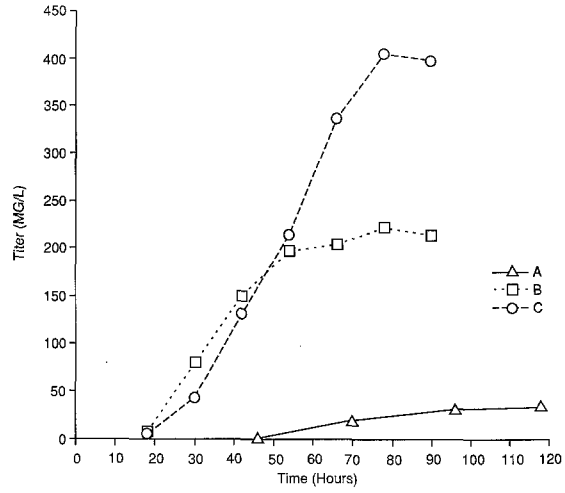

Fig. 2. Kinetics of asperlicin production in fermentors. Curve A: culture ATCC 20655, seed medium KF, production medium 2X LG; curve B: culture ATCC 20656, seed medium KF, production medium JK1; curve C: culture ATCC 20656, seed medium Syn 1, production medium Syn 2.

found after the original soil isolate was allowed to sporulate is shown in Fig. 1. The top three isolates from this experiment were used during the subsequent development of superior media. The isolate deposited as ATCC 20656 was the most consistently superior natural isolate.

The original production medium LG supported production of 15 – 30 mg/l of asperlicin. Early studies on the production of asperlicin in fermentors showed that enriching the LG production medium would result in higher titers. The medium used was 2XLG in fermentors and supported production of 35 mg/l after 118 h of incubation (Fig. 2, curve A).

The Plackett & Burman protocol is a statistical experimental design that allows the experimenter to test a large number of variables in a relatively small number of experiments [16]. This is accomplished by comparing the titer produced in a series of fermentation media when a variable is present to the titer produced in a series of fermentation media when the variable is absent. A comparison is made between that difference in titer to determine whether the variable appears to have a net positive or negative effect upon productivity. Internal controls as part of the experimental design can be used to calculate the statistical significance of the observation using the Student *t* square test.

We have used the Plackett & Burman protocol to rapidly screen complex medium ingredients that were beneficial to the production of asperlicin. Those ingredients showing a net positive effect upon titer were then used to formulate media following a modification of a Simplex protocol for medium optimization [2,15]. The results of this dual exercise are summarized in Tables 1 and 2.

Two media from the Plackett & Burman experiment supported the production of titers >50 mg/l. Medium PBS supported production of 57 mg/l of asperlicin. Medium PBG supported production of 60 mg/l of asperlicin. Following the Plackett & Burman protocol four ingredients – Ardamine pH, lard water, soybean meal and sodium citrate – appeared to support good production of asperlicin in complex media formulations (Table 1). Cod liver oil, $(NH_4)_2SO_4$, corn meal and KH_2PO_4 showed either small positive effects or minimal negative effects upon titer. These latter four nutrients were included along with the four significant ingredients in a Simplex array to provide nutritionally balanced media. Using these eight nutrients, Simplex media designated S1 – S9 (Table 2) were formulated. These nine media supported titers from 4 to 79 mg/l.

Table 1

Plackett-Burman variables

Variable	Test concentration		% Significance
	g/l	ml/l	
Ardamine pH	5.0		96
Lard water	5.0		83
Soybean meal	5.0		77
Sodium citrate	2.0		67
Cod liver oil		2.0	46
Polyglycol P2000		2.0	30
$(NH_4)_2SO_4$	2.0		29
Corn steep		5.0	21
Corn meal	10.0		–6
Tomato paste	5.0		–8
KH_2PO_4	2.0		–12
Dextrose	10.0		–15
Glycine	2.0		–17
Glycerol		10.0	–20
Pectin	10.0		–51
$CoCl_2 \cdot 6H_2O$	0.01		–80

Medium S8, which supported asperlicin production at 79 mg/l, became the base for another Plackett-Burman array. In this experiment supplements to medium S8 were tested. None of the supplements showed highly significant positive effects upon titer but one medium formulation, X9, supported titers of 90 mg/l.

Based upon the initial Simplex experiment, media formulations S10, S11 and S12 were tested. These media supported titers that would contribute to a gradual increase in average titer for all media tested. We have found that a gradual increase in mean titer is a characteristic of many Simplex optimization experiments. Fortunately, with asperlicin, the availability of new information suggested new variables for incorporation into the Simplex optimization program. Supplementation of a synthetic medium,

Table 2

Simplex media formulations

	Component[a] (g/l)												Titer[b] (mg/l)
	1	2	3	4	5	6	7	8	9	10	11	12	
S1	5.0	2.0	2.0	10.0	5.0	5.0	2.0	2.0	0	0	0	0	41
S2	0	5.0	0	25.0	10.0	0	1.0	0	0	0	0	0	4
S3	20.0	0	5.0	0	10.0	10.0	4.0	5.0	0	0	0	0	20
S4	20.0	1.0	2.0	5.0	10.0	0	0	10.0	0	0	0	0	22
S5	5.0	2.0	2.0	20.0	0	5.0	4.0	10.0	0	0	0	0	34
S6	10.0	2.0	0	0	20.0	20.0	0	5.0	0	0	0	0	38
S7	10.0	5.0	5.0	10.0	10.0	10.0	1.0	0	0	0	0	0	66
S8	5.0	2.0	1.0	5.0	5.0	20.0	2.0	2.0	0	0	0	0	79
S9	0	0	2.0	20.0	15.0	5.0	2.0	5.0	0	0	0	0	38
S10	12.8	0	3.3	3.0	9.2	12.8	2.2	6.7	0	0	0	0	18
S11	0	3.3	0	16.7	8.8	8.8	0	4.6	0	0	0	0	44
S12	0	2.8	0	13.7	8.8	15.2	2.7	1.0	0	0	0	0	64
S13	5.0	2.0	1.0	5.0	5.0	10.0	2.0	2.0	2.0	0	0	0	6
S14	5.0	2.0	1.0	5.0	5.0	10.0	2.0	2.0	1.0	2.0	0	0	180
S15	5.0	2.0	1.0	5.0	5.0	10.0	2.0	2.0	0.5	1.0	2.0	0	123
S16	5.2	2.1	1.6	10.1	8.5	12.5	1.8	3.8	0	0.3	0.2	0	75
S17	0	3.6	0.3	14.7	7.8	13.0	1.6	1.6	0.2	0.5	0.3	0	67
S18	3.8	2.5	1.5	6.5	12.2	16.6	0.8	0	0.3	0.4	0.3	0	106
S19	3.8	2.5	1.5	6.5	12.2	0	0.8	0	0.3	0.4	0.3	16.6	100
S20	5.0	2.0	1.0	5.0	5.0	0	2.0	2.0	1.0	2.0	0	20.0	220

[a] Component 1 = Ardamine pH, 2 = KH_2PO_4, 3 = $(NH_4)_2SO_4$, 4 = corn meal, 5 = lard water, 6 = soybean meal, 7 = cod liver oil, 8 = sodium citrate, 9 = anthranilic acid, 10 = L-phenylalanine, 11 = tryptophan, 12 = Pharmamedia.

[b] Asperlicin titer.

Medium III [B], with tryptophan increased asperlicin production from 5 mg/l to 35 mg/l. Phenylalanine also stimulated production when it was added to Medium III. At the same time the structure of asperlicin was reported [10] (Fig. 3). Tryptophan, leucine and anthranilic acid precursors could be inferred from the structure. Leucine had shown little effect upon asperlicin production in synthetic or complex media (date not shown) so only tryptophan, anthranilic acid and phenylalanine were added as ingredients in the Simplex experiments. Media S13, S14 and S15 were formulated that supported asperlicin titers from 6 to 180 mg/l. From these data medium JK1 was formulated for use in fermentors. In JK1 anthranilic acid and soybean meal were eliminated because of supply problems. Pharmamedia was added to replace these two ingredients. Kinetics of a batch in this medium that had a maximum titer of 223 mg/l are shown in Fig. 2, curve B.

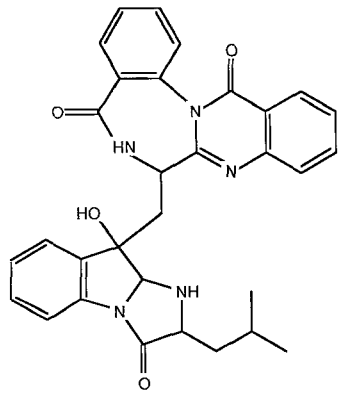

Fig. 3. Structure of asperlicin A.

Following up upon these observations, a series of

Table 3

Titration of glucose, glycerol, tryptophan and anthranilic acid in a synthetic medium[a]

Medium	Glucose (g/l)	Glycerol (g/l)	L-Tryptophan (g/l)	Anthranilic acid (g/l)	Asperlicin (mg/l)
1	10	0	1	0	19.7
2	10	0	1	1	32.8
3	10	0	5	0	59.3
4	10	0	5	1	43.9
5	20	0	1	0	8.9
6	30	0	1	0	4.4
7	0	10	1	0	255.8
8	0	20	1	0	153.2
9	0	30	1	0	65.0

[a] Medium Syn B plus ingredients listed.

synthetic media were formulated. Replacement of glucose with glycerol markedly altered titer. A medium with glucose produced 19.7 mg/l while replacement of glucose with glycerol resulted in a titer of 255.8 mg/l (Table 3). Further refinements in this synthetic medium resulted in the formulation of medium Syn 2, which in fermentors reached a peak titer of 408 mg/l (Fig. 2, curve C).

At this point, in the early development of the asperlicin fermentation, an economic issue was considered. What complex medium ingredient or ingredients could substitute for tryptophan and phenylalanine? Based upon its amino acid content, Amber Pro was selected for further study. Titers up to 500 g/l were observed in a complex medium without amino acid supplementation (Fig. 4).

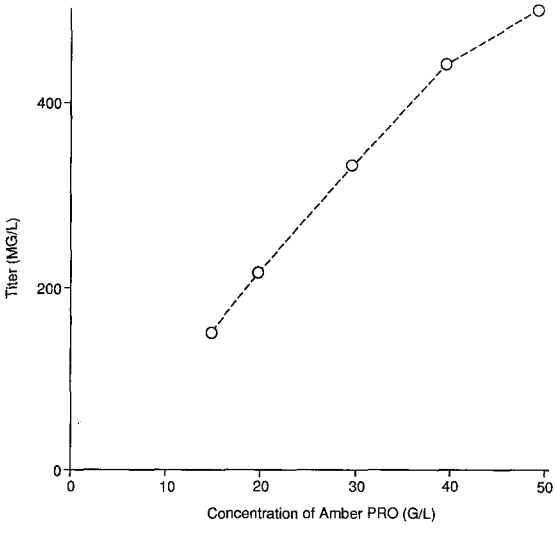

Fig. 4. Titration of Amber Pro in complex media. Medium EB33 with Amber Pro supplements from 15.0 to 50.0 g/l.

Table 4

Titration of phenylalanine and tryptophan in a complex medium

Medium[a]	Tryptophan (g/l)	Phenylalanine (g/l)	Asperlicin titer (mg/l)
1	0	8	178
2	1	8	469
3	2	8	628
4	3	8	780
5	4	8	839
6	4	0	261
7	4	2	514
8	4	4	629
9	4	6	694
10	4	8	818
11	4	10	913

[a] Medium AL 2PM plus ingredients listed.

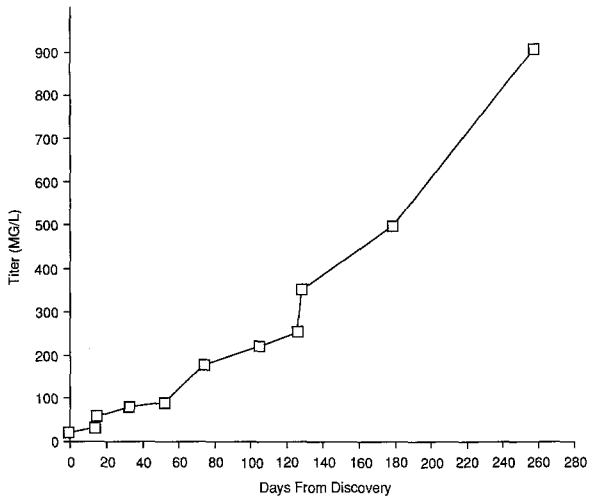

Fig. 5. History of asperlicin titer development.

Peak titers were obtained in a medium that combined much of what had been learned previously. Using glycerol as a carbon source and peptonized milk as a nitrogen source and titrating tryptophan and phenylalanine supplements titers up to 913 mg/l were obtained (Table 4).

The time had come to begin a mutation program. The initial mutation strategy was to select mutants resistant to amino acid analogs of tryptophan or phenylalanine. Among the initial mutants isolated was one capable of producing 20% more asperlicin than its parent. It was at this point in the program that development work on the asperlicin fermentation was suspended. Synthetic leads that evolved from the asperlicin discovery were to be further developed [3,4,6,14]. Fig. 5 summarizes the overall asperlicin development effort outlined in this report. For this product titer was increased from 15 – 30 mg/l to >900 mg/l in less than 9 months.

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