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Enzymatic cyclization of linear peptide to plant cyclopeptide heterophyllin B

JIA Aiqun[1], LI Xiang[2], TAN Ninghua[1], LIU Xiaozhu[3], SHEN Yuemao[1] & ZHOU Jun[1]

1. State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Graduate University of Chinese Academy of Sciences, Chinese Academy of Sciences, Kunming 650204, China;
2. Chinese Peptide Co., Ltd, Hangzhou 310018, China;
3. Southwest Forestry College, Kunming 650204, China

Correspondence should be addressed to Zhou Jun (email: jzhou@mail.kib.ac.cn)

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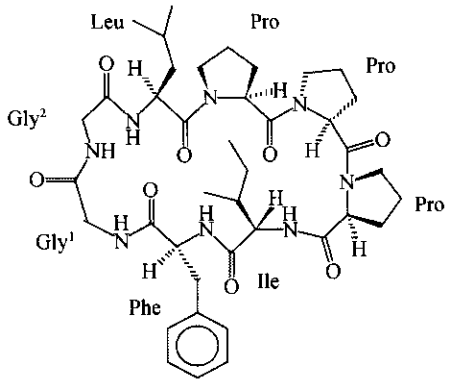
Abstract The crude enzyme (PH-**1**) isolated from *Pseudostellaria heterophylla* by our group has catalyzed enzymatic cyclization of linear peptide NH_2-Gly^1-Gly^2-Leu-Pro-Pro-Pro-Ile-Phe-COOH (**4**) into cyclopeptide heterophyllin B (HB) from plant for the first time. To ensure this reaction, some analytical methods including TLC, HPLC, MS, NMR, and ^{13}C labeling were used to prove that the reaction of substrate **4** sharing residue of NH-Phe-Gly-CO was successful.

Keywords: *Pseudostellaria heterophylla*, cyclopeptides, HB, enzymatic cyclization, isotope labeled.

Cyclopeptide is an important family of natural products. Over the past decade, about 100 cyclopeptides have been isolated from higher plants by our group[1]. The biosynthesis of cyclopeptides from microbes such as tyrocidine A has been reported recently[2−4]. The biosynthesis of cyclopeptides from higher plants, which has been puzzling us for a long time, has not been reported until now. In the effort to exploit a new enzymatic cyclization field in higher plant cyclopeptides, a crude enzyme (PH-**1**) extracted by us was used to determine whether this enzyme could catalyze some linear peptides into higher plant cyclopeptides in this communication.

The roots of *Pseudostellaria heterophylla* (Mig.) Pax ex Pax et Hoffm (Tai-zi-shen) are used as a well-known traditional Chinese medicine (TCM)[5]. Heterophyllin B (HB), of which the absolute configurations of all amino acid residues are ***L***s, is one of the main cyclopeptides in *P. heterophylla* isolated by our group[6]. We present here an enzymatic strategy to access the cyclization of HB. As it is a cyclic octapeptide, eight linear octapeptides would be candidate substrates in theory. Since there are three Pro residues in HB, only the following peptides **1**—**4** would be suitable precursors for the Pro's tension force:



Heterophyllin B (HB)

1: NH_2-Phe-Gly^1-Gly^2-Leu-Pro-Pro-Pro-Ile-COOH;
2: NH_2-Gly^2-Leu-Pro-Pro-Pro-Ile-Phe-Gly^1-COOH;
3: NH_2-Leu-Pro-Pro-Pro-Ile-Phe-Gly^1-Gly^2-COOH;
4: NH_2-Gly^1-Gly^2-Leu-Pro-Pro-Pro-Ile-Phe-COOH.

At first these linear octapeptides **1**—**4** were synthesized by solid-phase peptide synthesis (SPPS). Then PH-**1** was isolated from fresh *P. heterophylla* according to the process: pretreatment by cell broken and protein released, rough fractionation by dialysis and ultrafiltration, fine fractionation by molecular-exclusion chromatography, ion-exchange chromatography and lyophilization. Finally the enzymatic cyclizations of linear peptides **1**—**4** under PH-**1** were carried out as Scheme 1.

Scheme 1:

1: NH_2-Phe-Gly^1-Gly^2-Leu-Pro-Pro-Pro-Ile-COOH
2: NH_2-Gly^2-Leu-Pro-Pro-Pro-Ile-Phe-Gly^1-COOH
3: NH_2-Leu-Pro-Pro-Pro-Ile-Phe-Gly^1-Gly^2-COOH
4: GH_2-Gly^1-Gly^2-Leu-Pro-Pro-Pro-Ile-Phe-COOH

$\xrightarrow{\text{PH-1}}$ Heterophyllin B

Conditions: (a) Buffer: Tris: pH=8.0; (b) temperature: 24℃; (c) time: 3 h; (d) substrate **1**—**4**: Cylase=1:8; (e) yields: 27.28% for **4.**

By repeated reactions, we just found that linear peptide **4** was converted into HB in the yield of 27.28%, while other linear peptides **1**—**3** were negative under the same conditions. To ensure the positive reaction of **4**, the carbonyl carbon of N-terminal Gly^1 in **4** was labeled by ^{13}C as follows:

^{13}C labeled **4**:

NH_2-Gly^1-^{13}CO-Gly^2-Leu-Pro-Pro-Pro-Ile-Phe-COOH.

Under the same reaction conditions, the results indicated that ^{13}C labeled **4** could be converted into ^{13}C labeled HB too.

All the above results were proved by some analytic methods such as TLC, HPLC, MS and NMR.

Using authentic chemical detection methods for cyclopeptides[1], only HB and ^{13}C labeled HB were detected in the reaction solution of **4** after purification with purplish red spots on the TLC plate (Fig. 1).

After the reaction was finished, the HPLC analysis was carried out as shown in Fig. 2. The t_Rs (min) of **1**—**4** were 7.196, 12.533, 11.904, and 8.831, respectively, and the t_R (min) of **4**'s reaction products was 17.318 which was identical with the t_R of the authentic sample HB (17.340 min).

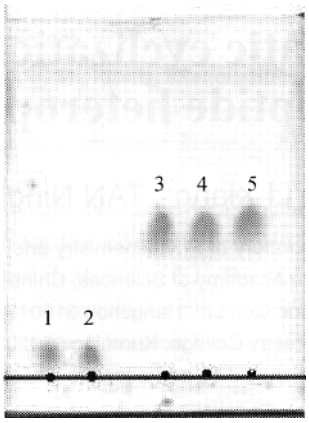


Fig. 1. TLC detection. (a) Solid phase: silical gel G; mobile phase: $CHCl_3$:CH_3OH 9:1. (b) 1, **4**; 2, ^{13}C labeled **4;** 3, ^{13}C labeled HB (by enzymatic cyclization); 4, HB (by enzymatic cylization); 5, HB (authentic sample).

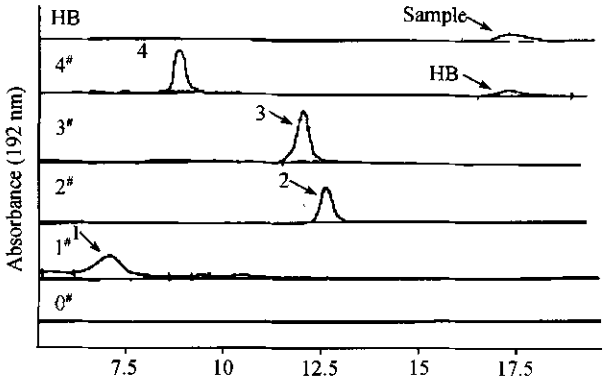


Fig. 2. HPLC detection. Instrument: Agilent 1100 series (Agilent Technologies, Wilmington, USA); column: Accurasil C-18 (5 μm, 250 ×4.6 mm); mobile phase: 70% H_2O and 30% CH_3CN; detection: 192 nm; flowing rate: 1 mL/min; $4^{\#}$ (3, 2, 1): enzyme+**4** (**3**, **2**, **1**); 0: enzyme.

Under the same mobile phase, the μg scale HB and ^{13}C labeled HB were obtained through semi-preparative HPLC for MS and NMR analysis.

As shown in Fig. 3, 778 and 779 were the respective molecular weights of HB and ^{13}C labeled HB with *m*/*z* 779: $[M+1]^+$ and 780: $[M+1]^+$ by FAB^+-MS.

To further test the cyclization of **4** successfully, the abundance of FAB^+-MS about substrates and reaction

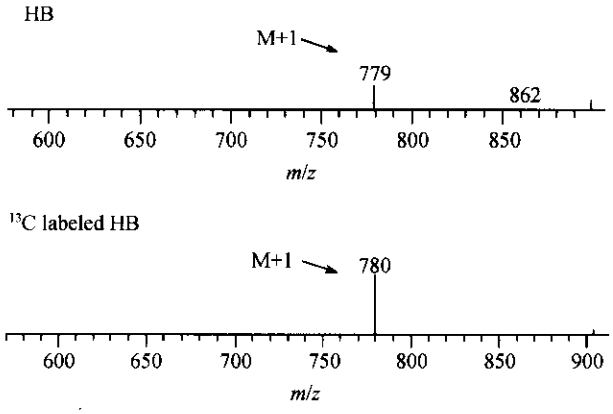


Fig. 3. FAB$^+$-MS spectra of HB and ^{13}C labeled HB.

production is shown in Table 1. The isotope composition of the enzymatic cyclized ^{13}C labeled HB was [^{13}C HB]/HB = 8:1 as determined by FAB$^+$-MS. The abundance of ^{13}C in the glycine[1] residue of ^{13}C labeled HB was 89.13%, while the abundance of ^{13}C in the glycine[1] residue of the substrate (^{13}C **4**) was 92.46%.

Further analysis was done by ^{13}C NMR. The ^{13}C NMR signal of 171.820 $\times10^{-6}$ was carbonyl of glycine[1] in HB by comparison with ref. [6] and 171.913 $\times10^{-6}$ was for carbonyl of ^{13}C HB which was downfield shifted by 0.093 $\times10^{-6}$ in C_5D_5N. But the chemical shift of the carbonyl of glycine[1] in ^{13}C labeled **4** was 169.846 $\times10^{-6}$ which was upfield shifted by 2.067 $\times10^{-6}$ (Fig. 4).

The above analysis shows that **4** can be successfully cyclized into HB under PH-1 extracted from *P. heterophylla*.

Before carrying out this experiment, we found that the residue NH - Gly – Phe - CO frequently appeared in cyclopeptides from higher plants by the statistical method. The results of reactions correspond with the statistical results that cyclopeptides may be cyclized primarily between Gly and Phe.

Our present research work has just localized to the enzymatic cyclization of HB. It is estimated that the enzymatic cyclization of other cyclopeptides sharing with the same residue NH - Gly – Phe - CO is possible.

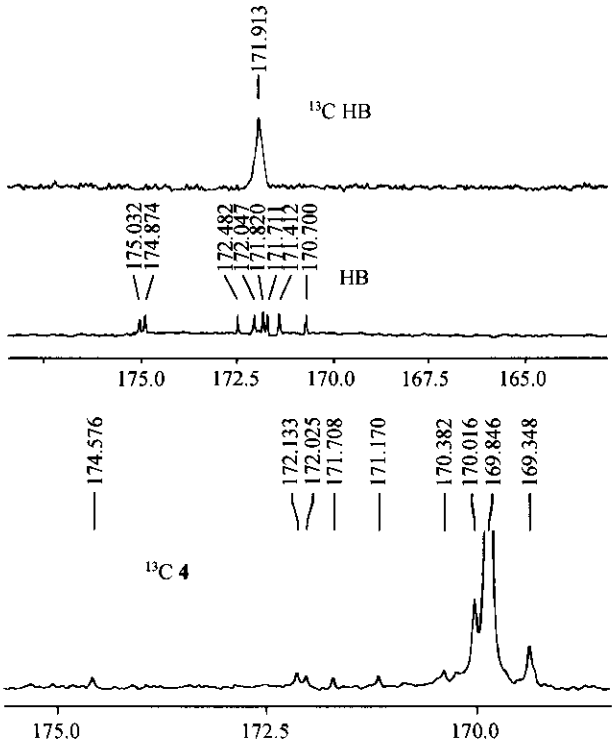


Fig. 4. ^{13}C NMR spectra of ^{13}C labeled **4**, HB and ^{13}C labeled HB (in C_5D_5N, 125 MHz).

The question of whether these enzymes are monoenzyme or multienzyme is also raised. Based on our experiments and biosynthetic results of microbe cyclopeptides[2−4], it appears that PH-**1** extracted from *P. heterophylla* might be multienzyme.

The cyclization of HB was the first enzymatic cyclization in the plant cyclopeptide field; while the background literatures have localized on the biosynthesis of cyclopeptides from microbes[2,3].

Table 1 The abundance of FAB$^+$-MS about ^{13}C labeled **4** and ^{13}C labeled HB

	M	M+1	M+2	M+3	M+4
^{13}C **4**	8.16	100.00	83.33	37.38	13.24
	(797)	(798)	(799)	(800)	(801)
^{13}C HB	12.19	100.00	83.11	14.36	7.13
	(779)	(780)	(781)	(782)	(783)

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作者单位： JIA Aiqun,TAN Ninghua,SHEN Yuemao,ZHOU Jun(State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Graduate University of Chinese Academy of Sciences, Chinese Academy of Sciences, Kunming 650204, China)， LI Xiang(Chinese Peptide Co., Ltd, Hangzhou 310018, China)， LIU Xiaozhu(Southwest Forestry College, Kunming 650204, China)

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