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COMMUNICATION

The *Botrytis cinerea* type III polyketide synthase shows unprecedented high catalytic efficiency toward long chain acyl-CoAs†

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BPKS from *Botrytis cinerea* is a novel type III polyketide synthase that accepts C_4–C_{18} aliphatic acyl-CoAs and benzoyl-CoA as the starters to form pyrones, resorcylic acids and resorcinols through sequential condensation with malonyl-CoA. The catalytic efficiency (k_{cat}/K_m) of BPKS was 2.8×10^5 s^{-1} M^{-1} for palmitoyl-CoA, the highest ever reported. Substrate docking analyses addressed the unique features of BPKS such as its high activity and high specificity toward long chain acyl-CoAs.

Type III polyketide synthases (PKSs) generate the backbones of a variety of plant secondary metabolites including chalcones, stilbenes, phloroglucinols, resorcinols, benzophenones, biphenyls, bibenzyls, chromones, acridones, pyrones, and curcuminoids.[1,2] The chalcone synthase (CHS) superfamily of type III PKS enzymes are homodimers of 40–45 kDa proteins that directly catalyze CoA-linked substrates using a single active site cavity.[3–5] The functional diversity of the CHS superfamily is attributable to differences in their selection of starter substrates, the number of polyketide chain extensions, and the mechanisms of their cyclization reactions.

The genomes of *Neurospora crassa*[6] and *Aspergillus oryzae*[7] predict the presence of type III PKSs in these filamentous fungi.[8] To date few fungal type III PKSs have been characterized, examples being 2-oxoalkylresorcylic acid synthase (ORAS) from *N. crassa*, CysA and CysB from *Aspergillus oryzae*[9–11] and AnPKS from *Aspergillus niger*.[12] To further examine the catalytic mechanisms of fungal type III PKSs and to use these enzymes for polyketide biosynthesis, additional fungal type III PKSs need to be identified. This work reports the characterization of the gene product (BPKS) of XM_001555277.1 from *Botrytis cinerea*. This is the first report that describes the catalytic properties of a type III PKS from *B. cinerea*, which shows the highest catalytic efficiency ever reported toward long chain acyl-CoA esters.

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Results

The amino acid alignment of BPKS with plant, bacterial and fungal type III PKSs showed conservation of the Cys-His-Asn catalytic triad in BPKS, evidence of BPKS being a type III PKS (Fig. S1, ESI†). Alignment of BPKS with other type III PKSs also revealed a carboxy-terminal extension of approximately 80 residues of BPKS. BPKS is longer than other type III PKSs known to have C-terminal extensions: 1,3,6,8-tetrahydroxynapthalene synthase (THNS) from *Streptomyces coelicolor*[13] and ORAS.[14] BPKS cDNA was cloned without C-terminal extension. The 1263 bp fragment of BPKS was cloned and expressed in *E. coli* BL21-CodonPlus (DE3)-RIL with an N-terminal 6×His affinity tag. The purified His_6-tagged BPKS protein gave a single protein band at ~45 kDa on SDS-PAGE (Fig. S2, ESI†). The truncation of BPKS did not affect the catalytic properties of the enzyme. The activity of the full length enzyme and the truncated version was almost the same (data not shown), which suggests that the 80 amino acids at the C-terminal extension are not essential for the enzymatic activity of BPKS. Similar findings were previously reported in other type III PKSs.[13,14]

Various starter-CoAs were tested for their reactivity toward BPKS. For C_2, C_4, C_6 and C_8 starter units, BPKS yielded a single product. For longer starters (C_{10} to C_{14}-CoA), two products with similar UV absorption profiles were produced. The HPLC profile of the product from hexanoyl-CoA (**2b**) showed a single major peak at 280 nm (Fig. S3A, ESI†). Upon LC/MS analysis, this product showed a molecular ion peak $(M - H)^-$ at m/z 181.1, attributable to a cyclized triketide (**3b**) synthesized from one molecule of hexanoyl-CoA and two molecules of malonyl-CoA. Triketide (**3d**) (M_r = 265.0) and tetraketide (**4d**) (M_r = 307.2) pyrones (Fig. S3B, ESI†) were formed from lauroyl-CoA (**2d**) by BPKS. Other than tri- and tetraketide pyrones, BPKS produced pentaketide resorcylic acids and resorcinols from the C_{16}–C_{18} starter-CoA substrates. A hexaketide resorcylic acid was also produced from C_{18}-CoA (**2g**). Fig. 1 shows the HPLC profile of the products synthesized by BPKS from C_{18}-CoA. The HPLC profiles of other starters are given in ESI.† Additionally, acetoacetyl-CoA and benzoyl-CoA

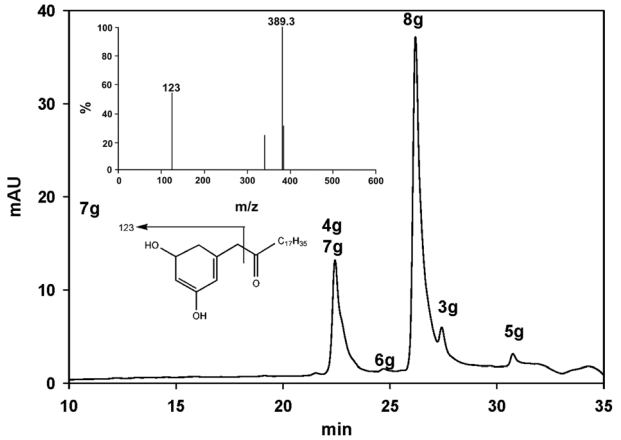

Fig. 1 HPLC analysis of the products synthesized by BPKS from stearoyl-CoA. The inset figures show the MS/MS spectra and chemical structure of the 5-(2′-oxononadecyl)-resorcinol (**7g**). MS/MS data of other major products (**3g**, **4g**, and **8g**) are given in ESI.†

were tested with BPKS. Acyl-CoA with an aromatic side chain, benzoyl-CoA (**2h**), could also be incorporated by BPKS, generating a triketide (**3h**, M_r = 188) (Fig. S3C, ESI†). Acetoacetyl-CoA (**2i**), whose structure mimics an intermediate in the polyketide elongation pathway, was also used by BPKS, yielding a triketide (**3i**, M_r = 126; Fig. S3D, ESI†). The exact molecular weights of all identified products were confirmed by HRMS (see ESI†). BPKS showed maximum activity with palmitoyl-CoA (based on its initial rate of free CoASH production) as a starter substrate. Table S1 (ESI†) lists the relative activity of BPKS with various starter CoAs. BPKS preferred long chain substrates for the synthesis of alkylresorcinols, while the α-pyrones were the major products of reactions primed with short and medium chain acyl-CoAs. The summary of reactions catalyzed by BPKS is shown in Fig. 2.

Starter unit specificity was investigated through steady state kinetic analyses of C_4–C_{18} acyl-CoAs (Table 1). Under optimal assay conditions (50 mM Tris-HCl, pH 8.0, and 30 °C), the highest k_{cat} (0.95 s^{-1}) and k_{cat}/K_m (2.8 × 10^5 s^{-1} M^{-1}) were observed for palmitoyl-CoA. K_m values of BPKS for hexanoyl-CoA and palmitoyl-CoA starters were 1.6 μM and 3.3 μM, respectively. K_m values of various type III PKSs for acyl-CoA starters range from 9.9 to 866.5 μM. Table S2 (ESI†) compares the catalytic properties of BPKS with those of other PKSs.[9,15–17] The specificity of BPKS toward long-chain aliphatic acyl-CoA (C_{14} to C_{18}) substrates is unprecedented in the fungal type III PKSs. ORAS did not accept CoAs with aromatic side chains whereas BPKS produced a triketide product with a catalytic efficiency of 4.5 × 10^3 s^{-1} M^{-1} using benzoyl-CoA as a starter unit. Among the fungal type III PKSs, next to AnPKS, BPKS showed the highest activity with the aromatic starter unit.

Previous work has shown that the active site cavity of type III PKSs acts as a size-based filter, determining starter substrate specificity, the number of possible extensions and the type of cyclization.[5] To investigate the structural basis for the observed substrate specificity, the homology model of BPKS was built based on the only crystal structure showing high sequence identity with BPKS: ORAS from *N. crassa* (PDB code 3E1H; 65% identity). The modeled structure of BPKS showed a similar acyl binding tunnel to that found in ORAS,

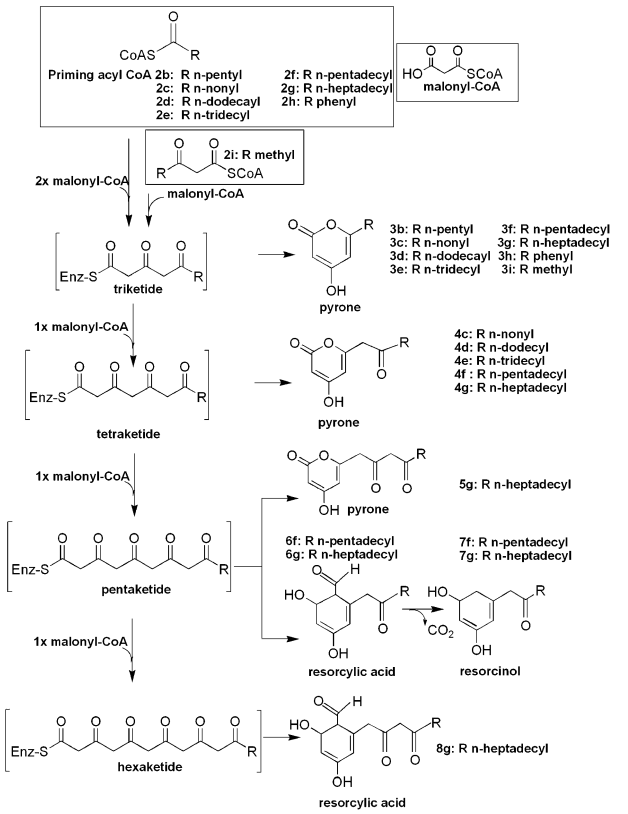

Fig. 2 Summary of BPKS reactions with various acyl-CoA starter substrates.

Table 1 Steady state kinetic parameters of BPKS protein with different starter units. Results are means (n = 3) with S.E. values less than 15%

Starter CoA	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
Acetoacetyl-CoA	0.04 ± 0.01	36.6 ± 1.5	1.1 × 10^3
Benzoyl-CoA	0.09 ± 0.01	19.8 ± 1.3	4.6 × 10^3
Butyryl-CoA	0.03 ± 0.01	3.8 ± 0.3	7.9 × 10^3
Hexanoyl-CoA	0.05 ± 0.01	1.6 ± 0.1	3.1 × 10^4
Decanoyl-CoA	0.10 ± 0.01	2.0 ± 0.1	5.0 × 10^4
Myristoyl-CoA	0.43 ± 0.02	3.3 ± 0.1	1.3 × 10^5
Palmitoyl-CoA	0.92 ± 0.05	3.3 ± 0.3	2.8 × 10^5
Stearoyl-CoA	0.75 ± 0.03	3.7 ± 0.4	2.0 × 10^5

RppA and PKS18, which has been reported to accommodate the binding of long chain acyl-CoAs.[13–15] When the modeled BPKS structure was superimposed onto the structure of ORAS, all the residues lining the active site cavity were well aligned; in particular, the catalytic triads, Cys-His-Asn (located at positions 165, 317, and 350, respectively, in BPKS), overlapped excellently. To investigate the molecular basis for the high activity of BPKS toward long chain substrates, stearoyl-CoA was docked into the substrate binding pocket of BPKS and ORAS, then variations in the orientations of catalytic residues between ligand bound BPKS and ORAS were examined. Fig. S4 (ESI†) shows the superimposition of BPKS modeled structure with bound stearoyl-CoA (C_{18} acyl-CoA) and ORAS structure co-crystallized with eicosanoic acid (C_{20} fatty acid). The acyl binding tunnel is lined by Phe265 (Phe252), Phe223 (Phe210), Cys133 (Cys120), Ser199 (Thr187) and Thr78 (Asn65) residues. It validates that the docked stearoyl-CoA in the active

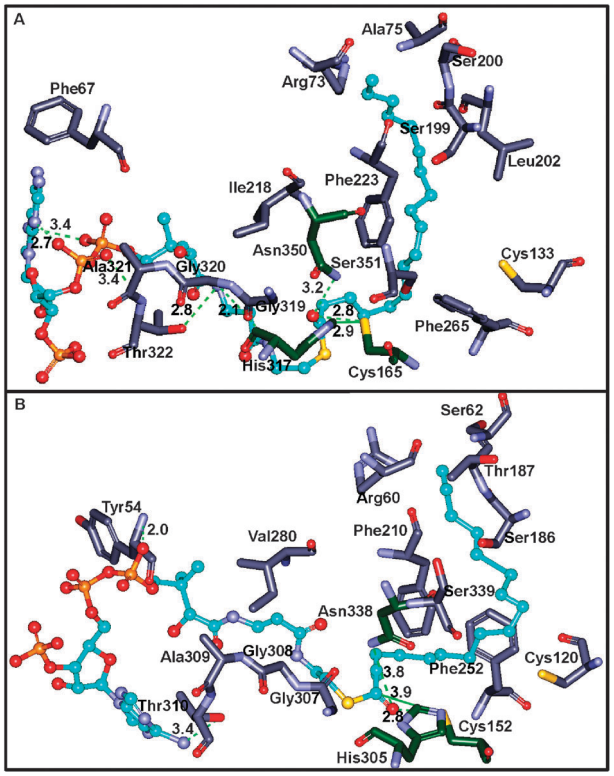

Fig. 3 Substrate docking of BPKS and ORAS in the substrate binding tunnel. (A) Stearoyl-CoA docked in the substrate binding tunnel of BPKS. (B) Stearoyl-CoA docked in the substrate binding tunnel of ORAS. Hydrogen bonds between stearoyl-CoA and amino acid residues are shown as green dotted lines. Stearoyl-CoA interactions with catalytic residues are shown as solid lines. Amino acid residues are shown in stick model colored grey while catalytic amino acid residues are shown with carbon in green color. Stearoyl-CoA is represented in ball and stick with carbon in blue color.

site pocket of BPKS modeled structure has a similar conformation to that of eicosanoic acid bound in ORAS.[14]

When stearoyl-CoA was docked into the substrate binding site of BPKS (Fig. 3A), stearoyl-CoA was bound through hydrogen bonds (green line) with Gly319 (2.1 Å), Ala321 (3.4 Å), and Thr322 (2.8 Å). A hydrogen bond (3.2 Å) has been observed between the thioester carbonyl oxygen and nitrogen of Asn350. While a distance of 2.9 Å was observed between the sulfur of the catalytic cysteine and the thioester carbonyl group of stearoyl-CoA which is essential to start the nucleophilic attack. Other intramolecular interactions were also seen. Fig. 3A shows that the residues Thr322, Ala321 and Gly319 contribute hydrogen bonds to CoA binding. All these interactions position the CoA molecule at the entrance to the BPKS active site and orient the C_{18} stearoyl moiety at the end of the CoA tunnel that opens into a cavity that defines the acyl binding tunnel. The other interactions observed between the bound stearoyl-CoA and the residues in the acyl-CoA binding tunnel are shown in Fig. S5 (ESI†). In ORAS, however, there was no hydrogen bond between oxygen of the phosphate and the Ala residue (Fig. 3B). Moreover, the distance of the thioester carbonyl group of stearoyl-CoA from the catalytic Cys152 was longer (3.9 Å) compared to the distance observed in BPKS. A hydrogen bond (3.8 Å) has been observed between the thioester carbonyl oxygen of stearoyl-CoA and Asn338. The CoA part of stearoyl-CoA formed hydrogen bonds only with Thr310 (3.4 Å) and Tyr54 (2.0 Å) residues. Palmitoyl-CoA was also docked into the substrate binding tunnel of BPKS. A hydrogen bond (2.7 Å) was observed between the thioester carbonyl oxygen and nitrogen of Asn350. A distance of 2.9 Å was observed between the sulfur of the catalytic cysteine and the thioester carbonyl group of palmitoyl-CoA. Several hydrogen bonding interactions were also observed (Fig. S6, ESI†). Previous studies on type III PKSs have shown that decreasing or increasing the number of CoA interactions may alter the kinetics of the association and dissociation of CoA thioesters and thus influence the fate of the type III PKS reaction intermediates.[5] The docking analyses support the high activity of BPKS toward palmitoyl-CoA and stearoyl-CoA.

Crystallographic and functional studies of CHS indicate that a cysteine–histidine pair (Cys164-His303) forms part of the catalytic machinery.[18] It is reported that the active site Cys164 nucleophile should be within the hydrogen bonding distance of His303 so that His303 Nε can form a stable imidazolium–thiolate ion pair with Cys164. In the crystal structure of CHS,[19] the Sγ of Cys164 formed a hydrogen bond (3.5 Å) with the Nε of His303. We determined the distance between Sγ of Cys (165 in BPKS and 152 in ORAS) and Nε of His (317 in BPKS and 305 in ORAS) in BPKS and ORAS structures. Distances of 3.4 Å and 3.8 Å were observed in BPKS and ORAS, respectively. This suggests that a more stable imidazolium–thiolate ion pair can be seen in BPKS (Cys165-His317) when compared to ORAS.

Pyrone structures have been found to be inhibitors, such as HIV-1 protease,[20] metalloprotein, human leukocyte elastase, and porcine pancreatic elastase,[21] making them unique lead structures for bioactive agents. Anti-cancer effects of alkylresorcinols have been elucidated in previous reports.[22,23] BPKS, as a type III PKS, exhibits the highest catalytic efficiency toward aliphatic CoAs than any other PKSs and thus is a potential biocatalyst for the synthesis of alkyl-pyrones and resorcinols. The product profiles can be controlled based on the length of the starter units.

Conclusions

In this study a putative type III PKS from *B. cinerea* was characterized. It synthesized fatty acyl-primed tri, tetraketide pyrones, resorcylic acid and resorcinol compounds from various starter units. Although BPKS displayed broad substrate specificity, it exhibited highest activity toward long chain length starter units. Homology modeling studies of BPKS with stearoyl-CoA shed light on the high activity of BPKS toward stearoyl-CoA. Crystal structure analyses combined with mutational studies are now in progress to probe the structural basis for the catalytic properties of BPKS.

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Notes and references

1 M. A. Fischbach and C. T. Walsh, *Chem. Rev.*, 2006, **106**, 3468–3496.
2 J. Staunton and K. J. Weissman, *Nat. Prod. Rep.*, 2001, **18**, 380–416.
3 S. Tropf, B. Karcher, G. Schroder and J. Schroder, *J. Biol. Chem.*, 1995, **270**, 7922–7928.
4 B. S. Moore and J. N. Hopke, *ChemBioChem*, 2001, **2**, 35–38.
5 M. B. Austin and J. P. Noel, *Nat. Prod. Rep.*, 2003, **20**, 79–110.
6 J. E. Galagan, S. E. Calvo, K. A. Borkovich, E. U. Selker, N. D. Read, D. Jaffe, W. FitzHugh, L. J. Ma, S. Smirnov, S. Purcell, B. Rehman, T. Elkins, R. Engels, S. Wang, C. B. Nielsen, J. Butler, M. Endrizzi, D. Qui, P. Ianakiev, D. Bell-Pedersen, M. A. Nelson, M. Werner-Washburne, C. P. Selitrennikoff, J. A. Kinsey, E. L. Braun, A. Zelter, U. Schulte, G. O. Kothe, G. Jedd, W. Mewes, C. Staben, E. Marcotte, D. Greenberg, A. Roy, K. Foley, J. Naylor, N. Stange-Thomann, R. Barrett, S. Gnerre, M. Kamal, M. Kamvysselis, E. Mauceli, C. Bielke, S. Rudd, D. Frishman, S. Krystofova, C. Rasmussen, R. L. Metzenberg, D. D. Perkins, S. Kroken, C. Cogoni, G. Macino, D. Catcheside, W. Li, R. J. Pratt, S. A. Osmani, C. P. DeSouza, L. Glass, M. J. Orbach, J. A. Berglund, R. Voelker, O. Yarden, M. Plamann, S. Seiler, J. Dunlap, A. Radford, R. Aramayo, D. O. Natvig, L. A. Alex, G. Mannhaupt, D. J. Ebbole, M. Freitag, I. Paulsen, M. S. Sachs, E. S. Lander, C. Nusbaum and B. Birren, *Nature*, 2003, **422**, 859–868.
7 M. Machida, K. Asai, M. Sano, T. Tanaka, T. Kumagai, G. Terai, K. Kusumoto, T. Arima, O. Akita, Y. Kashiwagi, K. Abe, K. Gomi, H. Horiuchi, K. Kitamoto, T. Kobayashi, M. Takeuchi, D. W. Denning, J. E. Galagan, W. C. Nierman, J. Yu, D. B. Archer, J. W. Bennett, D. Bhatnagar, T. E. Cleveland, N. D. Fedorova, O. Gotoh, H. Horikawa, A. Hosoyama, M. Ichinomiya, R. Igarashi, K. Iwashita, P. R. Juvvadi, M. Kato, Y. Kato, T. Kin, A. Kokubun, H. Maeda, N. Maeyama, J. Maruyama, H. Nagasaki, T. Nakajima, K. Oda, K. Okada, I. Paulsen, K. Sakamoto, T. Sawano, M. Takahashi, K. Takase, Y. Terabayashi, J. R. Wortman, O. Yamada, Y. Yamagata, H. Anazawa, Y. Hata, Y. Koide, T. Komori, Y. Koyama, T. Minetoki, S. Suharnan, A. Tanaka, K. Isono, S. Kuhara, N. Ogasawara and H. Kikuchi, *Nature*, 2005, **438**, 1157–1161.
8 Y. Seshime, P. R. Juvvadi, I. Fujii and K. Kitamoto, *Biochem. Biophys. Res. Commun.*, 2005, **331**, 253–260.
9 N. Funa, T. Awakawa and S. Horinouchi, *J. Biol. Chem.*, 2007, **282**, 14476–14481.
10 D. Yu, J. Zeng, D. Chen and J. Zhan, *Enzyme Microb. Technol.*, 2010, **46**, 575–580.
11 Y. Seshime, P. R. Juvvadi, K. Kitamoto, Y. Ebizuka and I. Fujii, *Bioorg. Med. Chem.*, 2010, **18**, 4542–4546.
12 J. Li, Y. Luo, J. K. Lee and H. Zhao, *Bioorg. Med. Chem. Lett.*, 2011, **21**, 6085–6089.
13 M. B. Austin, M. Izumikawa, M. E. Bowman, D. W. Udwary, J. L. Ferrer, B. S. Moore and J. P. Noel, *J. Biol. Chem.*, 2004, **279**, 45162–45174.
14 S. B. Rubin-Pitel, H. Zhang, T. Vu, J. S. Brunzelle, H. Zhao and S. K. Nair, *ACS Chem. Biol.*, 2008, **15**, 1079–1090.
15 P. Saxena, G. Yadav, D. Mohanty and R. S. Gokhale, *J. Biol. Chem.*, 2003, **278**, 44780–44790.
16 D. Yu, J. Zeng, D. Chen and J. Zhan, *Enzyme Microb. Technol.*, 2010, **46**, 575–580.
17 Y. Mizuuchi, Y. Shimokawa, K. Wanibuchi, H. Noguchi and I. Abe, *Biol. Pharm. Bull.*, 2008, **31**, 2205–2210.
18 J. M. Jez and J. P. Noel, *J. Biol. Chem.*, 2000, **275**, 39640–39646.
19 J. L. Ferrer, J. M. Jez, M. E. Bowman, R. A. Dixon and J. P. Noel, *Nat. Struct. Biol.*, 1999, **6**, 775–784.
20 C.-L. Sun, R.-F. Pang, H. Zhang and M. Yang, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 3257–3262.
21 R. W. Spencer, L. J. Copp and J. R. Pfister, *J. Med. Chem.*, 1985, **28**, 1828–1832.
22 A. Kozubek and J. H. Tyman, *Chem. Rev.*, 1999, **99**, 1–26.
23 A. B. Ross, A. Kamal-Eldin and P. Aman, *Nutr. Rev.*, 2004, **62**, 81–95.