Features of the biotechnologically relevant polyamide family “cyanophycins” and their biosynthesis in prokaryotes and eukaryotes

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Abstract
Cyanophycin, inclusions in cyanobacteria discovered by the Italian scientist Borzi in 1887, were characterized as a polyamide consisting of aspartic acid and arginine. Its synthesis in cyanobacteria was analyzed regarding growth conditions, responsible gene product, requirements, polymer structure and properties. Heterologous expression of diverse cyanophycin synthetases (CphA) in Escherichia coli enabled further enzyme characterization. Cyanophycin is a polyamide with variable composition and physiochemical properties dependent on host and cultivation conditions in contrast to the extracellular polyamides poly-γ-glutamic acid and poly-L-lysine. Furthermore, recombinant prokaryotes and transgenic eukaryotes, including plants expressing different cphA genes, were characterized as suitable for production of insoluble cyanophycin regarding higher yields and modified composition for other requirements and applications. In addition, cyanophycin was characterized as a source for the synthesis of polyanaspartic acid or N-containing bulk chemicals and dipeptides upon chemical treatment or degradation by cyanophycinases, respectively. Moreover, water-soluble cyanophycin derivatives with altered amino acid composition were isolated from transgenic plants, yeasts and recombinant bacteria. Thereby, the range of dipeptides could be extended by biological processes and by chemical modification, thus increasing the range of applications for cyanophycin and its dipeptides, including agriculture, food supplementations, medical and cosmetic purposes, synthesis of the polyacrylate substitute polyanaspartic acid) and other applications.

Introduction
Cyanophycin, which is also referred to as cyanophycin granule polypeptide (CGP), has been the subject of research for over 40 years. Beginning with pioneering studies in cyanobacteria in the early 1970s, including first characterization of the synthesizing enzyme, heterologous expression in other bacteria, yeasts and higher plants to engineer the CGP composition and enhancing cellular contents for large scale production, CGP research is leading to consideration of the first commercial pilot projects, and the polymer became better known. Therefore, this review provides an overview of the history of CGP research, insights in the improvement of CGP content in industrially relevant strains, the systematic modification of the CGP constituents synthesized in prokaryotes and eukaryotes as well as application of CGP and its derivatives in agriculture, medicine, cosmetic, nutrition and degradable biopolymers.

Historical landmarks
CGP in filamentous nitrogen-fixing cyanobacteria
CGP was discovered as light scattering inclusions by the Italian scientist Borzi during microscopic observations of cyanobacteria. It took some time until data on the chemical composition and structure of this compound, and its function and biosynthesis were published (Table S1). Biochemical pioneer work was mainly performed by Robert D. Simon who published first data about a reserve material consisting of a copolymer of aspartic acid and arginine isolated from the filamentous nitrogen-fixing blue-green algae Anabaena cylindrica in 1971. Moreover, he described the isolation, chemical and molecular mass determinations of CGP granules. The isolated granules were characterized by SDS-PAGE and gel chromatography as polymers with molecular masses between 25 and 100 kDa. Arginine and aspartic acid were detected in a molar ratio of 1:1 by different chemical methods.

Therefore, it was proposed that such a polymer could be a suitable storage protein for nitrogen and synthesis of
oxaloacetate. Additionally, these granules were characterized as insoluble in distilled water and as soluble in dilute acid or base. Synthesis of CGP was determined as a function of A. cylindrica culture age, starting at the end of the growth phase, it was utilized when growth resumes. Thus supporting the assumption that CGP is a storage compound (Simon, 1973a).

Simon et al. (1973b) demonstrated that chloramphenicol inhibited growth and production of large amounts of CGP in an exponentially growing culture. Hence, it was concluded that CGP was synthesized non-ribosomally. In 1976, the CGP synthesizing enzyme of A. cylindrica was purified 92-fold, and in vitro synthesis of CGP was analyzed by Simon et al. (1973b) for the first time. The enzymatic reaction was dependent on the presence of MgCl₂, KCl, ATP and a sulfhydryl reagent. Furthermore, de novo synthesis was dependent on an arg-(poly-asp) primer or additional unknown enzymes or factors. The L-arginine analogue canavanine was characterized as an inhibitor of the incorporation reaction of arginine (Simon, 1976).

CGP isolated from A. cylindrica was characterized by different spectroscopic methods. It was shown that multivalent L-arginy1-poly(L-aspartic acid) (Simon & Weathers, 1976) can exhibit an ordered structure which is dependent of the incubation conditions. It was proposed that the acid soluble and neutral insoluble forms of CGP have similar conformations and contain some fractions of β-pleated sheet structure (Simon et al., 1980).

In the filamentous nitrogen-fixing cyanobacterium Anabaena sp. PCC 7120, the cyanophycin synthetase was more active in heterocysts than in vegetative cells. The reaction was also dependent on a CGP primer, and the synthesized polymer consisted of aspartic acid and arginine (Gupta & Carr, 1981) as also in Anabaena variabilis ATCC 29413 (Ziegler et al., 1998).

In nitrogen-fixing Scyttonema species, glycogen and CGP accumulation were reported as a consequence of salinity stress (Page-Sharp et al., 1998). The filamentous nitrogen-fixing cyanobacterium Nostoc ellipso sporum also accumulated CGP consisting of aspartic acid and arginine in vegetative cells and akinetes during stationary growth (Leganes et al., 1998).

**CGP in unicellular non-nitrogen-fixing cyanobacteria**

Structure and composition of CGP₆₃₀₈ of the unicellular, non-nitrogen fixing cyanobacterium Synechocystis (Aphanocapsa) 6308 were characterized by Allen & Weathers (1980). As demonstrated for A. cylindrica, no granule could be isolated from cells grown under optimal conditions in the logarithmic phase. Light, CO₂, sulfur and phosphorus starvation as well as the addition of arginine to the culture medium, increased the amount of CGP₆₃₀₈ when compared with cells grown under optimal growth conditions (Allen et al., 1980). Furthermore, these granules, consisting of aspartic acid and arginine, were catabolized during cultivation in low-CO₂ concentrations (Allen & Weathers, 1980). Therefore, it was concluded that CGPs isolated from nitrogen-fixing or non-nitrogen-fixing cyanobacteria were similar regarding chemical composition, granule fine structure and function as storage compounds.

Synthesis of CGP₆₈₀₃ in the unicellular cyanobacterium Agmenellum quadruplicatum and Synechocystis sp. PCC 6803 was also investigated (Stevens & Paone, 1981; Ziegler et al., 1998). In contrast to these two cyanobacteria, a significant variation of the amino acid composition of CGP₆₃₀₈ as a function of growth conditions was demonstrated for Synechocystis sp. 6308 by Merritt et al. (1994). Here, nitrogen limitation resulted in a polymer consisting of an aspartic acid backbone and glutamyl side chains. Therefore, glutamic acid decreased and arginine increased when nitrate was provided to nitrogen-limited cells.

In comparison to the aforementioned strains, no CGP could be isolated from three marine and two freshwater strains of the genus Synechococcus (Newman et al., 1987). When Hai et al. (1999) analyzed Synechococcus sp. MA19, a thermophilic species of this genus, regarding CGP synthesis, a polymer composed of aspartic acid and arginine occurring at a molar ratio 1:0.9 could be purified, after reducing light intensity and temperature plus the addition of arginine and chloramphenicol. The molecular mass of this especially polydisperse polymer ranged from 20 to 130kDa with a maximum at 50kDa. A deviating composition was determined for CGP₆₂₁ purified from nitrogen-limited Synechococcus sp. G2.1 cultivated with given amounts of nitrate and chloramphenicol. The polymer consisted of 35 mol% aspartic acid, 29 mol% of arginine, 15 mol% glutamic acid and 21 mol% of a wide variety of amino acids (Wingard et al., 2002). Finding glutamic acid in CGP₆₂₁ confirmed the previous results of Merritt et al. (1994) for Synechocystis sp. PCC 6308.

In summary, the composition of CGP accumulated in cyanobacterial cells mostly consisted of aspartic acid and arginine (Table S2). Synthesis of CGP was described as a function of the growth phase, light, available nutrients and temperature. It was characterized as suitable storage compound for nitrogen, carbon and energy.

**CGP content in cyanobacterial strains**

Even though CGP was first described in cyanobacteria, these organisms will not be used for production for several reasons. Besides very long cultivation times of 14 or more days, they only achieve very low cell densities in comparison to E. coli or Ralstonia eutrophica and require a photo-bioreactor as well as excessive process control. Exact measurements of the CGP content for most of these cyanobacteria are scarce. Initially, CGP was often determined using a method established by Messineo (1966), which measures CGP by determination of the arginine concentration in relation to the chlorophyll content of the cells (mg Arg x mg Chl⁻¹) thereby providing relative data only comparable to closely related strains. In addition, these strains synthesize CGP as a storage compound and therefore possess enzymes capable of degrading CGP which could reduce the measurable maximal CGP content. Therefore, data of reported CGP content in strains capable of degrading CGP must be carefully discussed.

Synechocystis PCC 6308, which possesses one of the most used CphAs (CphA₆₃₀₈), was determined to synthesize CGP₆₃₀₈ in amounts up to 16.0% of the cellular dry mass (CDM) under optimized cultivation conditions.
CGP in non-cyanobacterial wild-type strains

In addition to the discovery of CGP in several cyanobacteria, genomes of non-cyanobacteria were analyzed regarding the occurrence of genes for CGP biosynthesis. Several genes homologous to cphA encoding the cyanophycin synthetase of Synechocystis sp. PCC 6803 were identified in strains of Acinetobacter, Bordetella, Clostridium botulinum, Nitrosomonas europaea, Desulfitobacterium hafniense and Ralstonia eutropha H16 (Adames et al., 2013; Krehenbrink et al., 2002; Ziegler et al., 2002). In case of Acinetobacter sp. DSM 587, a mutant of A. baylyi ADP1, CGP$_{ADP1}$ was accumulated under phosphate-limited conditions during the stationary growth phase. This polymer also consisted of equimolar amounts of aspartate and arginine, and the apparent molecular mass ranged from 21 to 28 kDa, which is lower than the masses of CGP typically isolated from cyanobacteria (Krehenbrink et al., 2002).

In summary, the composition of CGP accumulated in cyanobacterial and non-cyanobacterial cells mostly consisted of aspartic acid and arginine (Table S2). Synthesis of CGP was described as a function of the growth phase, light, available nutrients and temperature. It was characterized as a suitable storage compound for nitrogen, carbon and energy.

Molecular and biochemical insights in CGP synthesis

Non-ribosomal peptides and biotechnological relevant poly(amino acids)

Non-ribosomal peptides (NRPs), unlike proteins, are synthesized independent of messenger RNA and often have cyclic and/or branched structures. They can also contain non-proteinogenic amino acids or those carrying modifications like N-methyl and N-formyl groups, or are acylated, glycosylated or hydroxylated. This complexity and structural versatility is achieved by NRP synthetases (NRPSs), large multimodular enzyme complexes composed of an array of distinct sections each of which is responsible for the incorporation of one defined monomer into the final peptide (Fischbach & Walsh, 2006). While synthesis of CGP as well as of the two other naturally occurring poly(amino acids), poly(glutamic acid) and poly(lysine), shares the template-independent synthesis and resistance against translation inhibitors (e.g. chloramphenicol) with NRP synthesis, they differ in two essential characteristics: poly(amino acids) are synthesized by comparatively simple enzymes and are composed of only one or two types of amino acids in their backbone, thus lacking the complex structure and versatility of NRPs. The template-independent synthesis results in a remarkable size distribution and polydispersity of the polymers, while homodisperse NRPs are all of identical structure and size since the multimodular structure of the NRPSs serves as a replacement for a template. Among poly(amino acids), CGP shows another difference due to the pendant arginine residues linked to the β-carboxyl groups of the aspartyl residues (Simon & Weathers, 1976), while poly(lysine) and poly(glutamic acid) possess only an amino acid backbone.

Characterization of cyanophycin synthetases

First investigations on CGP biosynthesis were performed by Simon (1976), who identified the CGP synthesizing enzyme of A. cylindrica, characterized as a non-ribosomal peptide synthetase and named cyanophycin synthetase (CphA). Purifications of active CphA proteins from various wild-type and recombinant strains unraveled allowed substrate specificity and affinity, cofactors and other properties. CphA proteins isolated and purified directly from the wild-type strains include the enzymes of A. cylindrica (Simon, 1976), A. variabilis ATCC 29413 (Ziegler et al., 1998) and Synechococcus sp. strain MA19 (Hai et al., 1999), while other CphA proteins were isolated from recombinant E. coli strains expressing the corresponding cphA. These include the synthetases of A. variabilis ATCC 29413 (Berg et al., 2000), Synechocystis sp. PCC 6308 (Aboulmagd et al., 2001a) and A. baylyi ADP1 (Krehenbrink & Steinbüchel, 2004).

The optimum activity of CphA$_{6308}$ in terms of temperature is around 50 °C but leading to fast inactivation of the enzyme within 30 min. However, for optimal function temperatures, 28–30 °C exhibited prolonged stability with reasonable enzyme activity, while basic pH at 8.2 is required for optimal function and the activity drops significantly below a pH of 6 (Aboulmagd et al., 2001a; Krehenbrink & Steinbüchel, 2004; Simon, 1976; Simon & Weathers, 1976). Polymer synthesis depends on ATP, L-arginine and L-aspartic acid, a primer, catalytic amounts of Mg$_2^+$, K$^+$ and a sulfhydryl reagent such as β-mercaptoethanol (Aboulmagd et al., 2001a; Simon, 1976).

Molecular characterization of CphA from A. variabilis was conducted by Ziegler et al. (1998). The molecular mass of the native enzyme was estimated to be 230±30 kDa by chromatography, while the denatured enzyme exhibits a molecular mass of 100 kDa in SDS-PAGE. Therefore, a homodimeric nature of the enzyme was proposed. Since it was demonstrated that ATP was converted to ADP during the CphA enzyme assay, some amino acid residues were predicted to be part of an ATP binding site (Ziegler et al., 1998).

The lengths of CphA synthetases extend from 873 amino acids (Synechocystis sp. PCC 6308) to 914 amino acids (A. baylyi strain ADP1). Analyses of truncated CphA proteins from N. ellipsosporum strain NE1 revealed that presumably only the first 870 amino acids are relevant for enzyme activity (Hai et al., 2006).
The first cyanophycin synthetase gene (cphA) was identified in the completely sequenced genome of *Synechocystis* sp. PCC 6803 on the basis of six partial amino acid sequences determined for the CphA of *A. variabilis* (Ziegler et al., 1998). Additional molecular characterization were carried out by Berg et al. (2000), aligning the amino acid sequences of CphAs from two filamentous and two unicellular cyanobacteria. In addition, Ziegler et al. (1998) detected two putative active sites and another ATP-binding site, located in the N-terminal region. This ATP-site showed similarity to a superfamiliy of ATP-dependent ligases including carboxylate-amine and carboxylate-thiol ligases (Galperin & Koonin, 1997). Characteristic of these enzymes is the phosphorylation of carboxylates with an Mg$^{2+}$-ATP complex and an ATP-grasp fold type possessing a B- and J-loop in its binding site (Hibi et al., 1996). Sequence analysis of the C-terminus demonstrated a similarity to a superfamiliy of ATP-dependent ligases including carboxylate-amine and carboxylate-thiol ligases (Galperin & Koonin, 1997). Characteristic of these enzymes is the phosphorylation of carboxylates with an Mg$^{2+}$-ATP complex and an ATP-grasp fold type possessing a B- and J-loop in its binding site (Hibi et al., 1996). Sequence analysis of the C-terminus demonstrated a similarity to a superfamiliy of peptide ligases from *E. coli* which are involved in peptidoglycan biosynthesis. This family also includes FolC, a polyglutamate synthetase-dihydrofolate synthetase (Eveland et al., 1997; Sun et al., 1998).

**Mechanism of CGP synthesis**

Ziegler et al. (1998) showed that during catalysis, ATP is converted to ADP and phosphate; AMP is not formed. Therefore, it was concluded that the amino acid substrates are activated by phosphorylation of their carboxyl groups. The elongation mechanism of the CphA reaction was proposed by Berg et al. (2000) after it was discovered that a suitable primer was elongated by the enzyme at its C-terminus. The proposed catalytic reaction cycle includes the addition of one dipeptide unit to the C-terminus of a CGP primer in four successive steps (Figure 1). First, the carboxylic group of the polyaspartic acid backbone is activated by transfer of the γ-phosphoryl group of ATP, and one molecule of aspartic acid is bound at the C-terminus of the CGP-primer by its amino group, forming a peptide bond. Afterwards, a second molecule of ATP is hydrolyzed, leading to the linkage of arginine to the primer through an isopeptide bond. This synthesis cycle corresponds well with structural analysis of the protein.

Low amounts of CGP or an oligopeptide consisting of at least three Asp-Arg dipeptides [(β-Asp-Arg)$_3$] are required as a primer for maximal enzyme activity (Berg et al., 2000). The origin of this primer in heterologous strains is unclear but in *vitro* the purified CphA still shows a low activity of 1–3% even in the absence of added primers. Therefore, it is likely that this base activity is used to synthesize the first few dipeptides which then serve as primer, successively increasing the activity of the enzyme. Another discussed possibility is that di-, tri- or tetrapeptides of various amino acids occurring from protein degradation inside the cells might serve as substitute primers to ignite CGP synthesis until it is replaced by the newly synthesized CGP.

Interestingly, CphA from *T. elongatus* BP-1 was identified as a primer-independent CphA (Arai & Kino, 2008). What exactly causes independency from a primer is not yet verified, but *in silico* analysis of the putative primer binding region of the enzyme shows slight variations in the amino acid sequence of CphA_{BP-1} in comparison to primer-dependent
CphA proteins like CphA<sub>6308</sub>, CphA<sub>7120</sub> and CphA<sub>MA19</sub> (Figure S1). Since the primer binding region is only putative, effects of these variations are arguable.

**In vitro substrate specificity of CphA proteins**

Several CphA proteins were purified from recombinant *E. coli* strains and characterized regarding substrate specificity (Table S2). As mentioned above, arginine and aspartate were determined as constituents of accumulated CGP isolated from different cyanobacterial and non-cyanobacterial strains. CphA purified from *Synechocystis* sp. PCC6308 (CphA<sub>6308</sub>) incorporated *in vitro* instead of arginine canavanine and up to 15 mol% lysine but no glutamate (Aboulmagd et al., 2001a, b; Krehenbrink et al., 2002). Incorporation of glutamate instead of arginine could only be demonstrated *in vitro* with crude extracts (Table S2). An incorporation of canavanine instead of arginine (Table S2) could also be demonstrated for CphA<sub>MA19</sub> purified from recombinant *E. coli* (Hai et al., 2002). CphA<sub>29413</sub> accepted also other basic amino acids like lysine, ornithine and citrulline instead of arginine (Berg et al., 2000). The non-cyanobacterial CphA from *A. baylyi* ADP1 was partially purified from recombinant *E. coli* and characterized (Krehenbrink & Steinbüchel, 2004) and did not accept lysine as alternative substrate to arginine. This enzyme had a slightly higher affinity for aspartic acid when compared to cyanobacterial CphA (Krehenbrink & Steinbüchel, 2004).

CphA from the thermophilic cyanobacterium *T. elongatus* BP-1 could use Mg<sup>2+</sup> instead of Mn<sup>2+</sup> without loss of activity (Arai & Kino, 2008). The activity could be increased by KCl but not by DTT. The pH optimum was 9.0 and the protein showed thermal stability by retaining 80% activity after a 60-min incubation at 50°C. For industrial CGP production *in vivo* or *in vitro*, thermal stability is a favorable property. Furthermore, the yield of CGP<sub>BP-1</sub> is not limited by the amount of a primer, as this is the case of all other previously presented CphAs. In contrast to *in vivo* synthesized CGP<sub>BP-1</sub>, *in vitro* synthesized CGP<sub>BP-1</sub> exhibits a narrower molecular mass distribution although it is still polydisperse. Additionally, this enzyme was also characterized as specific for the incorporation of aspartate and arginine (Arai & Kino, 2008). The characterization of other CphA proteins also demonstrated that only aspartate and arginine were incorporated *in vitro* (Table S2).

**Composition and yield of CGP in recombinant strains**

After *in vitro* characterization of CphA enzymes, the next sections will focus on the *in vivo* synthesis of CGP. From this, the significance of recombinant strains expressing *cphA* becomes obvious due to higher yields and variations of CGP in comparison to wild-type strains.

**Compositions and yields of insoluble CGP occurring in recombinant bacteria**

Research with recombinant strains started only after 1998 with expression of *cphA* from *A. variabilis* ATCC 29413, *Anabaena* sp. strain PCC 7120, *Synechocystis* sp. strain PCC 6803, *Synechocystis* sp. strain PCC 6308, *S. elongatus* and *Synechococcus* sp. strain MA19 in different strains of *E. coli* (Aboulmagd et al., 2000; Berg et al., 2000; Hai et al., 1999; Oppermann-Sanio et al., 1999; Ziegler et al., 1998) followed by expression studies also in *C. glutamicum*, *R. eutropha* and *P. putida* (Aboulmagd et al., 2001b). Depending on the recombinant/transgenic organism and cultivation conditions, CGP accumulated with different amino acid composition, to greater contents and with partially changed solubility behavior in comparison to cyanobacteria.

**Heterologous expression of cphA in *E. coli***

Heterologous expression of *cphAs* firstly occurred in *E. coli* reported by Ziegler et al. (1998) expressing *cphA<sub>6803</sub>* in *E. coli* DH5α. The synthesized CGP<sub>6803</sub> had a higher electrophoretic mobility than CGP<sub>6803</sub> isolated from *Synechocystis* sp. PCC 6803, and the material contained, in addition to aspartic acid and arginine, some lysine (Table S4). Polymeric material could also be isolated from recombinant *E. coli* cells expressing *cphA<sub>6308</sub>*. HPLC analysis revealed equimolar amounts of aspartate and arginine; with a range of 20 to 40 kDa average molecular mass. The polydispersity of CGP<sub>6308</sub> was lower in comparison to the CGP<sub>6308</sub> isolated from *Synechocystis* sp. PCC 6308 which exhibited a molecular mass of up to 100 kDa. It was proposed that the absence of an additional unknown catalytic factor, an insufficient supply of substrates or an unfavorable substrate/ enzyme ratio were in *E. coli* responsible for the differences which could also explain the inability of the recombinant enzyme to incorporate L-glutamic acid (Aboulmagd et al., 2000).

Berg et al. (2000) reported an incorporation of up to 10 mol% lysine besides aspartate and arginine in CGP<sub>29413</sub> from *E. coli* expressing *cphA<sub>29413</sub>* cultivated in double-strength LB at 25°C (Table S4) and concluded that rapid accumulation of CGP may deprive the cells of L-arginine, thus favoring the incorporation of L-lysine. Krehenbrink et al. (2002) also reported that CGP<sub>6308</sub> isolated from *E. coli* expressing *cphA<sub>6308</sub>* contained 10 mol% of lysine besides aspartic acid and arginine (Table S4). In contrast to the result of Aboulmagd et al. (2000), who detected no lysine in CGP<sub>6308</sub> isolated from recombinant *E. coli*, Krehenbrink & Steinbüchel (2004) used a different *E. coli* strain, and cultivation was accomplished at 30°C instead of 37°C. Furthermore, they found that expression of the non-cyanobacterial *cphA<sub>ADP1</sub>* in *E. coli* resulted in CGP<sub>ADP1</sub> synthesis as well.

After identification of recombinant bacteria being suitable for biotechnological production, Frey et al. (2002) optimized the isolation of CGP<sub>6803</sub> applicable on technical scale while also confirming the results of Ziegler et al. (1998) regarding lysine incorporation in CGP<sub>6803</sub> from *E. coli* strains expressing *cphA<sub>6803</sub>* in modified cultivation conditions resulting in CGP<sub>6803</sub> consisting of aspartate, arginine and lysine (1:0.47:0.32). Another study confirmed that recombinant *E. coli* expressing *cphA<sub>6803</sub>* contains CGP<sub>6803</sub> composed of 50 mol% aspartate, 46 mol% arginine and 4 mol% lysine (Table S4) (Elbahloul et al., 2005b).

Polymer isolated from recombinant *E. coli* expressing *cphA<sub>MA19</sub>* in LB at 37°C was composed of aspartate and
arginine at a molar ratio of 1:0.98 (Hai et al., 2002), while cphA from N. ellipsosporum truncated at the C-terminus, produced in E. coli CGP_{NE1} with only traces of lysine. Moreover, it was proposed that this CphA exhibited a higher substrate specificity than other cyanobacterial CphA proteins (Hai et al., 2006). CGP produced in E. coli by differently modified CphAs showed little glutamic acid and lysine or only lysine (1–4 mol%), in addition to aspartic acid and arginine, respectively (Hai et al., 2009).

No CGP-like material could be isolated from E. coli expressing a CphA homologue from D. hafniense, but in contrast to a negative control strain, crude extracts exhibited in SDS polyacrylamide gels typical polydisperse areas ranging from 30 to 35 kDa. This polymer was characterized as water-soluble, and a suitable isolation procedure was established by Ziegler et al. (2002). The molar composition of this polymer regarding aspartic acid, arginine and lysine was at a ratio of 1:0.9:0.1 (Table S4). This polymer was characterized as CGP-similar material with an altered solubility behavior. It was suggested that water-solubility must be caused by rather subtle differences in structure and/or composition (Ziegler et al., 2002).

**Heterologous expression of cphA in different bacteria**

The functional activity of CphA_{6308} in other bacteria like C. glutamicum, R. eutropha and P. putida was first examined by Aboulnagd et al. (2001b). In all cases, equimolar amounts of aspartic acid and arginine were detected, and the isolated material was identified as CGP (Table S4). It was also concluded that R. eutropha and P. putida were suitable candidates for the biotechnological production of CGP due to high productivity on mineral salt media. The isolated polymers also exhibited a slightly reduced length and higher polydispersity as it was previously reported for recombinant E. coli.

Voss et al. (2004) confirmed these results by isolating insoluble CGP from recombinant strains of R. eutropha, C. glutamicum, P. putida and Bacillus megaterium expressing cphA_{6308}. Moreover, the authors determined that CGP_{6308} contained up to 10 mol% lysine as substitute for arginine in the side chain of the polymer, when recombinant P. putida and R. eutropha strains were cultivated in mineral salt medium without any supplements. Furthermore, it was also concluded that the Gram-positive bacteria were not suitable for production of CGP (Table S5). In addition, first results of heterologous expression of cphA_{6803} and cphA_{AMA19} in R. eutropha and P. putida and also of cphA_{7120} cultivated at 30°C in mineral salt medium were presented, thus expanding the variety of CphAs available for CGP synthesis. CGP isolated from recombinant E. coli, in contrast to recombinant R. eutropha and P. putida, contained minor amounts of lysine besides aspartate and arginine (Voss et al., 2004).

The heterologous expression of cphA_{7120} in a bacterium, which infects leguminous plants resulting in a nitrogen-fixing symbiosis yielded a polymer composed of aspartate and arginine and a molecular mass distribution from 20 to 25 kDa in recombinant cells of Sinorhizobium meliloti (Table S4). No variations in the composition of the CGP_{7120} occurred despite cultivation of recombinant strains in a medium supplemented with different combinations of amino acids (Abd-El-Karem et al., 2011).

In summary, recombinant bacteria expressing cphAs were characterized as suitable sources for production of CGP composed of aspartate and arginine with up to 10 mol% lysine. The accumulated polymers showed a higher electrophoretic mobility than CGP from cyanobacteria and, except for the unique result of Ziegler et al. (2002), were characterized as insoluble. Moreover, E. coli seemed to be interesting for the synthesis of more heterogenic polymers due to the incorporation of lysine by CphA_{6308}, CphA_{6803} and CphA_{29413} (Table S4). In contrast, CphA_{7120}, CphA_{ADP1}, CphA_{AMA19} and CphA_{NE1} were more substrate specific, due to the lack of lysine in CGP isolated from E. coli strains. Additionally, R. eutropha and P. putida were identified as host for the synthesis of insoluble CGP without lysine. This was presented for recombinant CphA_{6803} and CphA_{AMA19} with the exception of CphA_{6308}.

**Compositions and yields of soluble CGP occurring in recombinant organisms**

While E. coli and R. eutropha were considered for commercial production of insoluble CGP, the interest in the use of other organisms including eukaryotes like transgenic plants, yeasts or other prokaryotes like P. putida ATCC4359 is driven by proof of concept for CGP synthesis and search of CGP variations (Figure 2) with changed solubility behavior or new constituents and implementation of these discoveries on the established production strains.

**Synthesis of CGP in transgenic plants**

Transgenic plants expressing a cyanophycin synthetase were first constructed by Neumann et al. (2005) by introducing and expressing CphA of T. elongatus BP-1 under a 35S promoter in tobacco and potato plants. Even though the first results showed some negative effects like reduced seed production and impaired growth and only showed contents of approximately 0.7–1.1% CGP in the CDM of tobacco and potato leaves (Table S5), synthesis of insoluble and to a smaller content soluble CGP_{BP-1} in higher eukaryotes was demonstrated. A maximum size of 35 kDa was determined by SDS-PAGE, and the soluble form was composed of aspartate, arginine and lysine in a molar ratio of 1:1.05:0.1 (Table S4). Different strategies were applied to enhance the productivity of plants which increased the CGP_{BP-1} content to 6.8% of CDM in leaves of specific tobacco plants and 7.5% of CDM (Table S5) in potato tubers without significant disturbance of plant growth and development by targeting the primer-independent CphA_{BP-1} to the chloroplasts (Hühns et al., 2008, 2009). Due to the fact that the first experiments in plants were performed in 2005 by Neumann et al. and in 2008 by Hühns et al., before the discovery of the primer independency of CphA_{BP-1} by Arai & Kino (2008), it is unlikely that this property was decisive for the use of CphA_{BP-1}. Furthermore, to this date, CphA_{BP-1} is the only reported CphA used in plants. However, primer independency might be essential for CGP synthesis in plants.
Synthesis of CGP in transgenic yeasts

The heterologous expression of cphA\textsubscript{6308} in yeasts also resulted in two forms of CGP\textsubscript{6308}. Extensive work regarding the establishment of CGP synthesis and the synthesis of CGP with different side chains in transgenic Saccharomyces\textit{cerevisiae} and\textit{Pichia pastoris} was conducted by Steinle et al. (2008, 2009, 2010). First expression experiments of cphA\textsubscript{6308} in\textit{S. cerevisiae} demonstrated that a majority of soluble polymer and a minority of insoluble polymer were accumulated. SDS-PAGE revealed molecular mass distributions from 20 to 35 kDa for the polydisperse soluble CGP\textsubscript{6308} and from 26 to 45 kDa for the polydisperse insoluble CGP\textsubscript{6308}. No differences regarding the amino acid composition were detected. Both forms consisted mainly of the two amino acids aspartic acid and arginine and in addition a minor amount of lysine (2 mol\%, Table S4). The content of lysine could be increased by cultivation of the transgenic yeast in the presence of 15 mM lysine (Steinle et al., 2008). The knowledge of arginine metabolism of\textit{S. cerevisiae} enabled the production of novel cyanophycins with an extended range of constituent amino acids. By using strains defective in arginine degradation and synthesis CGP\textsubscript{6308} containing citrulline and ornithine were synthesized by expressing cphA\textsubscript{6308}. CGP\textsubscript{6308} consisting of 20 mol\% citrulline, 8 mol\% ornithine or 16 mol\% lysine in addition to aspartate and arginine (Table S4, Figure 2) were synthesized by transgenic ARG1, ARG3 and ARG4 mutants. Furthermore, it was demonstrated that incorporation of citrulline or ornithine can be enhanced by addition of low amounts of arginine and also of L-citrulline or L-ornithine, respectively, to the medium. CGP synthesis was also established in two different strains of the methylo trophic\textit{P. pastoris} by heterologous expression of cphA\textsubscript{6308} and mutated cphA\textsubscript{6308}. As presented for\textit{S. cerevisiae}, two forms of CGP\textsubscript{6308} were isolated from transgenic\textit{P. pastoris} GS115 and\textit{P. pastoris} KM71H and characterized by SDS-PAGE and HPLC. Insoluble CGP\textsubscript{6308} isolated from both strains consisted of 3.5 mol\% lysine in addition to aspartate and arginine (Table S4). The compositions of soluble polymers isolated from both strains differed regarding incorporated amounts of lysine. Fractions of 4.7–6.8 mol\% were detected in soluble CGP\textsubscript{6308} isolated from transgenic\textit{P. pastoris} GS115, and a fraction of 14 mol\% lysine was detected in soluble polymer isolated from transgenic\textit{P. pastoris} KM71H (Table S4). The molecular mass distribution of the insoluble CGP\textsubscript{6308} isolated from transgenic\textit{P. pastoris} GS115 ranged from 24 to 35 kDa and of the soluble CGP\textsubscript{6308} from 19 to 40 kDa. After establishment of CGP synthesis in transgenic yeasts, Steinle & Steinbüchel (2010) established also an efficient, time-saving and cost-effective method for isolation of soluble CGP from yeast cells. Maximal CGP\textsubscript{6308} yields of 21\% (w/w) of CDM were obtained after incubation of freeze-dried cells from\textit{S. cerevisiae} at 70–80°C and precipitation of the polymer with two volumes of ethanol. No differences in molecular size distribution were detected in comparison to the results obtained by following the isolation procedure of

![Chemical structures of dipeptides–constituents of CGP detected upon \textit{in vivo} (A–E) and \textit{in vitro} (a–b). (A) Aspartate–arginine, (B) aspartate–lysine, (C) aspartate–citrulline, (D) aspartate–ornithine, (E) aspartate–glutamate. (a) Aspartate–canavanine, (b) aspartate–homoarginine, (c) aspartic acid β-methyl ester derivative of aspartic acid incorporated \textit{in vitro}.](image-url)
Ziegler et al. (2002) for recombinant E. coli (Steine & Steinbüchel, 2010).

**Synthesis of CGP in transgenic fungi**

The filamentous fungus *Rhizopus oryzae* 99-880 is another eukaryote for soluble CGP. An auxotrophic mutant was selected to express *cphA*6803 and *cphA*7120, and enzyme activity was detected in a transformant expressing *cphA*6803. Two polymer forms were isolated and characterized. The soluble form was the major fraction and accounted merely 0.5% of the CDW, whereas the insoluble form occurred only in traces. The soluble CGP6803 consisted of equimolar amounts of aspartate and arginine (Table S4) (Meussen et al., 2012). The molecular mass of insoluble and soluble CGP6803 showed a very similar mass distribution ranging from 20 to 37 kDa with only slight variations between different strains, as revealed by SDS-PAGE.

**Synthesis of CGP in recombinant *P. putida* ATCC 4359**

Nine years after Ziegler et al. (2002) demonstrated a water-soluble CGP6803 in a recombinant *E. coli* strain expressing *cphA*6803, soluble CGP was synthesized in another recombinant bacterium, *P. putida* ATCC 4359 is able to synthesize large amounts of citrulline from L-arginine and was therefore used as a bacterium. *P. putida* ATCC 4359 is able to synthesize large amounts of amino acids incorporated besides aspartate (Ziegler et al., 2002). The use of double-strength LB medium had slight effects on the ratio of soluble and insoluble forms but not on the compositions of both polymers (Tseng et al., 2012). This was different to the results of Steine et al. (2009), where addition of ornithine or citrulline to the medium resulted in their increased incorporation into CGP.

Further experiments with recombinant *E. coli* strains expressing four different *cphA*s confirmed that an increased lysine content is the main characteristic of soluble CGP (Frommeyer & Steinbüchel, 2013). Cultivation of the cells for 72 h in TB medium at 30 °C yielded both polymers independent of the origin of CphA. Therefore, this study confirmed the results of Tseng et al. (2012) with CphA6803 regarding increased incorporation of lysine in soluble CGP and showed, for the first time, that soluble CGP contained at least 17 mol% lysine beside aspartate and arginine synthesized by *E. coli* strains expressing *cphA*6308, *cphA*7120 or *cphA*ADP1 (Table S4). The recombinant strain expressing *cphA*6308 was especially interesting for synthesis of large amounts of soluble CGP6308 (23% CDM) composed of aspartate, arginine and lysine in a molar ratio of 1:0.5:0.5 (Table S4). The molecular masses of soluble CGP isolated from the four different recombinant strains ranged from approximately 12 to 40 kDa. The molecular masses of insoluble CGP isolated from recombinant *E. coli* were 20 to 35 kDa when using recombinant *E. coli* strains harboring cyanobacterial CphA6308. CphA6803 and CphA7120 or 25 to 170 kDa when harboring non-cyanobacterial CphAADP1 (Frommeyer & Steinbüchel, 2013).

**Conclusions regarding the solubility and non-solubility of CGP**

The occurrence of soluble beside insoluble CGP was new as a soluble form was never described previously in cyanobacterial cells. The findings regarding composition and solubility behavior were puzzling: on the one hand, heterologous expression of *cphA* in eukaryotes demonstrated the occurrence of two polymers with differences regarding yield or molecular mass distribution. Differences in amino acid composition of both CGP forms were demonstrated for mutated *S. cerevisiae* and two different *P. pastoris* strains (Steine et al., 2009) but not for transgenic plants, fungi or wild-type yeasts. On the other hand, the results of heterologous expression of *cphA*s in prokaryotes demonstrated differences regarding molecular mass, yield and amino acid composition of both forms. An incorporation of 17 mol% lysine or lysine plus citrulline in combination was responsible for the change of solubility behavior. This was demonstrated for recombinant *P. putida* and for four different recombinant *E. coli* strains (Frommeyer & Steinbüchel, 2013; Wiefel et al., 2011). These results were contrary to results by Ziegler et al. (2002) where only soluble CGP6803 was isolated from *E. coli*. Füser and Steinbüchel (2005) whether in vitro solubilization of insoluble CGP occurred in solutions of inorganic salts including NaCl. Ziegler et al. (2002) used a double-strength LB medium where high amounts of NaCl likely enabled the isolation of soluble CGP with a minor content of lysine from recombinant *E. coli* strain.
Degradation of CGP

Degradation of CGP by cyanobacterial cyanophycinase (CphB)

In 1980, an exopeptidase activity was observed in extracts of heterocysts of two N₂-fixing Anabaena species. This cyanophycinase exhibited a pH optimum of 8.5, did not require monovalent or divalent cations, and was inhibited by L-arginine and L-aspartic acid. The product of this enzyme was an aspartic acid–arginine dipeptide (Gupta & Carr, 1981). Furthermore, in cell extracts of non-N₂-fixing unicellular Synechocystis sp. PCC 6308 protease activity was also discovered. The pH optimum was 8.0, and optimal temperature was 35°C, and protease activity was also inhibited by aspartic acid and arginine. An assay was developed for measuring CGP hydrolysis (Allen et al., 1984). A cyanophycinase gene was identified in the sequenced genome of Synechocystis sp. PCC6803 by heterologous expression in E. coli (Richter et al., 1999). This gene (cphB) codes for a protein of 29.4 kDa, which appears to be specific for CGP. Hydrolyzed the polymer to dipeptides consisting of aspartate and arginine.

Degradation of CGP by extracellular cyanophycinases

After identification of eubacteria growing on CGP as sole carbon source, it was concluded that these strains must possess an enzyme specialized for CGP degradation (Obst et al., 2002). Subsequently, a strain of Pseudomonas anguilliseptica was isolated, and an extracellular cyanophycinase (CphE) was purified and characterized. The enzyme exhibited only 27–28% amino acid sequence identity to intracellular cyanophycinases occurring in cyanobacteria. A high specificity for CGP was exhibited, and degradation products were also identified as aspartate-arginine dipeptides (β-Asp-Arg) by HPLC and ESI-MS (Obst et al., 2002). Further cyanophycinases from Gram-positive bacteria were isolated and characterized (Obst et al., 2004, 2005). Hydrolysis of CGP with CphE from B. megaterium BAC19 yielded tetrapeptides beside dipeptides. A novelty was the complete turnover of CGP under anaerobic conditions by a consortium consisting of a strictly anaerobic, Gram-positive endospore-forming bacterium Sedimentibacter hongkongensis and the Gram-negative enterobacterium Citrobacter amalonaticus (Obst et al., 2005). Moreover, another CGP degrading strain was isolated and characterized from an anaerobic consortium consisting of four strains (Sallam & Steinbüchel, 2008). This study demonstrated CGP degradation under anaerobic and aerobic conditions by one strain, characterized as Pseudomonas alcaligenes strain DIP1. CGP was hydrolyzed to its constituting β-asp-arg dipeptides, which were completely hydrolyzed to alanine, succinate and ornithine (Sallam & Steinbüchel, 2008). A triphasic process consisting of synthesis and optimized acid extraction for technical scale isolation of CGP from biomass, fermentative production of extracellular cyanophycinase (CphEal) from P. alcaligenes DIP1 at 500 l scale, and degradation of CGP to β-asp-arg and β-asp-lys dipeptides with purity over 99% by employing a crude CphEal preparation was developed (Sallam et al., 2009). Furthermore, CphEal was characterized as serine protease with maximum activity at 50°C and at pH 7 to 8.5 (Sallam et al., 2009).

Production of CGP

Different strategies for CGP production at large scale were established. A maximum CGP content of 28% (wt/wt) CGP6803 per CDM was obtained in 6% (vol/vol) protamylase medium, utilizing a residual compound of starch production at an initial pH of 7.0 within a cultivation period of only 24 h (Elbahloul et al., 2005b). In terms of CGP synthesis, the establishment of plasmid addiction systems was successful. Since R. eutropha exhibits overall low-plasmid stability, even while supplied with antibiotics, the high-plasmid stability provided by the eda-addiction system made it possible to cultivate R. eutropha for a prolonged period. Amounts of CGP6308 exceeding 40% of CDM (Table S5) (Voss & Steinbüchel, 2006) represented a drastic improvement in comparison to previous fermentations or flask cultivation experiments, where comparable results were only possible with excessive and uneconomic use of antibiotics and supplementation.

Another plasmid addiction system (dapE/L-system) was developed for large-scale production of CGP C595S with E. coli (Kroll et al., 2011). The plasmid harbors cphA C595S, a cphA308 derivative with enhanced catalytic activity. Furthermore, a new mineral salt medium was designed for enhanced CGP production. CGP C595S with a molecular mass in a range from 26 to 35 kDa and with a lysine content of nearly 6 mol% was obtained (Table S4). Table S5 provides an overview of CGP contents reached in recombinant organisms over the past 13 years.

Applications of CGP

The chemical structure and composition of CGP offer different possibilities for applications. CGP synthesis is efficient due to the establishment of different platforms like recombinant microorganisms or transgenic plants, combined with suitable expression systems for production in larger scale. However, applications of CGP as a whole polymer are currently unknown.

Polymeric derivatives of cyanophycins

Since a homopolymer consisting of aspartic acid is part of the CGP backbone, copolymers of polyaspartic acid (PASP) with

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reduced arginine content or even PASP homopolymer could theoretically be obtained from CGP by mild chemical or by enzymatic hydrolysis (Joentgen et al., 2001). PASP is a water-soluble and biodegradable polymer with interesting properties permitting application in different fields. One application is as an anti-scalant, because scale deposition is a difficulty encountered with water containing ions of sparingly soluble salts, which strike cooling water systems, water desalination processes and oil field operations (Hasson et al., 2011). Further, similar applications for PASP as an additive in laundry detergents, dishwashing or as dispersants for a variety of organic and inorganic solids were suggested (Schwamborn, 1997) or bioflocculation treatment of industrial wastewater using PASP (Zhang et al., 2009). Biomedical applications could be achieved by polymeric hydrogels, which exhibit high-water absorbency, biodegradability, potential non-toxicity and biocompatibility (Kim et al., 2002). Furthermore, PASP could be applicable for biodegradable plastics (Conrad, 2005).

Since soluble lysine-rich CGP was identified, a novel possibility for the synthesis of a new polyamide by chemical modification became available. Thereby, lysine residues are transformed to homoarginine (2-amino-6-guanidinohexanoic acid) by reaction with o-methylisourea (OMIU) under alkaline conditions (Imbeah et al., 1996). The complete conversion of available functional amino groups to guanidino groups is limited to 5 min at 65 °C (Frommeyer et al., 2014). The resulting polymer, designated as modified CGP$_{595S}$ (mCGP), consisted of 50 mol% aspartic acid, 15 mol% arginine and 35 mol% homoarginine. Homoarginine (Figure 2) was never detected before as CGP constituent in cyanobacteria or recombinant microorganisms expressing cphAs.

Dipeptides and other products from cyanophycins

It was demonstrated by Hellier et al. (1972) that constituent amino acids were absorbed faster in the intestine of humans when presented as dipeptides instead of free amino acids, suggesting intact dipeptide transport. Meredith et al. (1990) confirmed that peptide diets are better absorbed than whole-protein diets in patients after trauma. The biodegradability of CGP by gut flora of several mammalian, avian and fish provides a first confirmation for the potential of CGP and its dipeptides in nutrition and therapy as highly bio-available sources for arginine, lysine, aspartate and possibly all other amino acids (Sallam et al., 2009; Sallam & Steinbüchel, 2010). Santos et al. (2012) proposed dipeptides and tripeptides as very appealing for drug discovery and development because of their cost-effectiveness, possibility of oral administration, and simplicity to perform molecular structural and quantitative structure–activity studies.

CGP is also a potential source for conversion to several N-containing bulk chemicals e.g. 1,4-butanediamine and acrylonitrile, 1,4-butanediol or urea (Mooibroek et al., 2007; Sanders et al., 2007).

Outlook

A biopolymer with the structure of CGP offers some interesting possibilities for applications as mentioned before. The isolation of soluble CGP$_{595S}$ with increased lysine content of up to 35 mol% by heterologous expression of a mutated CphA raises the question if it is possible to synthesize also a polyamide consisting of aspartic acid and lysine as sole constituents. As shown by Wiefel & Steinbüchel (2014) insoluble CGP$_{595S}$ can be separated by temperature depending on the polymer’s lysine content. If a similar separation could be performed with soluble CGP, which is most likely a mixture of polymer chains with different lysine contents as well, CGP with lysine contents even higher than 35–37 mol% could be purified. Since lysine is an essential amino acid for humans, CGP dipeptides consisting of aspartic acid and lysine are more interesting for commercial uses than dipeptides consisting of aspartic acid and arginine. Processes for enhancing synthesis and for optimizing the purification of CGP with increased amounts of lysine are highly relevant for these applications since the separation of a desired dipeptide gets progressively easier the higher its fraction in the dipeptide mixture is.

Another possible use for lysine-rich CGP is the chemical modification of the polymer. As demonstrated for the conversion of lysine to homoarginine by Frommeyer et al. (2014), the ε-amino group of lysine is accessible to chemical modification. By proving the feasibility of a chemical modification of one of the side chains of the polymer, a whole new spectrum of alternative modifications should be considered as an option. Modification of other alternative constituents like citrulline and ornithine or arginine should also be considered.

Declaration of interest

The authors report no declarations of interest.

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Supplementary material available online
Supplementary Table S1–S5, Figure S1.