Solubility Behavior of Cyanophycin Depending on Lysine Content

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Study of the synthesis of cyanophycin (CGP) in recombinant organisms focused for a long time mostly on the insoluble form of CGP, due to its easy purification and its putative use as a precursor for biodegradable chemicals. Recently, another form of CGP, which, in contrast to the insoluble form, was soluble at neutral pH, became interesting due to its high lysine content, which was also assumed to be the reason for the solubility of the polymer. In this study, we demonstrate that lysine incorporated into insoluble CGP affected the solubility of the polymer in relation to its lysine content. Insoluble CGP can be separated along a temperature gradient of 90°C to 30°C, where CGP showed an increasing lysine content corresponding to a decreasing temperature needed for solubilization. CGP with less than 3 to 4 mol% lysine did not become soluble even at 90°C, while CGP with 31 mol% lysine was soluble at 30°C. In lysine fractions at higher than 31 mol%, CGP was soluble. The temperature separation will be suitable for improving the downstream processing of CGP synthesized in large-scale fermentations, including faster and more efficient purification of CGP, as well as enrichment and separation of dipeptides and CGP with specific amino acid compositions.

Cyanophycin [multi-1-arginyl-poly(1-aspartic acid)], often abbreviated CGP (cyanophycin granule polypeptide), is a non-ribosomally synthesized biopolymer, originally discovered in cyanobacteria (1) but also naturally synthesized by a variety of photo- and heterotrophic bacteria for nitrogen, carbon, and energy storage (2, 3, 4). CGP is interesting due to its easy and low-cost purification using alternating steps of solubilization in 0.1 M HCl and precipitation by neutralization to pH 7 (5). Also of interest is the possibility of obtaining CGP with different side chains in which 1-arginine is replaced by other compounds, like lysine (6), ornithine (7), or citrulline (7, 8). Synthesis of these forms of CGP became possible because the amino acids mentioned also possess a certain affinity for the cyanophycin synthetase CphA (EC 6.3.2.29 and EC 6.3.2.30). This increases the possible applications of CGP or its dipeptides (9, 10) and allows the production of novel bulk chemicals (11, 12, 13).

Recently, it became evident, that in most studies aiming at the incorporation of large fractions of the alternative constituents into CGP, these compounds were found in a soluble form of CGP, while the insoluble form contained only small amounts of the constituents. Steinele et al. (7), for example, synthesized soluble CGP with a citrulline content of over 20 mol%, while the corresponding insoluble form contained citrulline only at a fraction of about 5 mol%. Unlike the normal insoluble CGP, which can only be solubilized in weak acids, like 0.1 M HCl, and which is insoluble at neutral pH, the soluble CGP is still soluble at pH 7.0 and can only be isolated by precipitation using ethanol (EtOH) or acetone (4). Analyses of the soluble polymer by nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), and α-phthalaldehyde (OPA) derivatization showed no differences in its composition or in the basic structure in comparison to the insoluble form, except for an increased content of alternative constituents. Whether these increased amounts of constituents are responsible for the solubility of CGP or whether the solubility behavior of CGP caused these modifications, i.e., by modification of the polymer after synthesis, remained unclear.

Over the years, several theories about the causes of these changes in solubility behavior were discussed. Some theories centered on changes in the structure of the CGP formed due to changes of the milieu in the cells, since soluble CGP often occurred in cells of recombinant strains not grown at their normal temperature. Pseudomonas putida, for example, synthesized soluble CGP only when cultivated at an elevated temperature of 37°C, while insoluble CGP was formed only at the usual growth temperature of 30°C (8). Several studies on Escherichia coli showed that synthesis of soluble CGP occurred only at a temperature of 30°C or less, while cells of the same strain grown at 37°C contained much less or no soluble CGP (6, 14). Another theory suggested that the increased amounts of alternative constituents influenced the solubility or the structure of CGP, or both (6). Also, combinations of these theories were suggested (6). Having an explanation for the solubility behavior might also be helpful in improving the downstream processing and purification of CGP and the correlated dipeptides. However, it was never clear how these theories could be proven in an appropriate manner. Since temperature was likely to be a factor influencing the solubility of CGP, in this study, we performed a simple solubilization test of crude, nearly unpurified Lys-rich insoluble CGP from E. coli.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions. All bacterial strains and plasmids used in this study are listed in Table 1. Strains of E. coli were grown at 30°C or 37°C in Erlenmeyer flasks or in 25-liter bioreactors using terrific broth (TB) (15). Unless otherwise indicated, lactose was used as the single or an additional carbon source. Strains possessing antibiotic resistance were provided with 100 µg/ml ampicillin and 50 µg/ml kanamycin in the medium. The strain used for CGP synthesis includes an addiction system that increases plasmid stability by deletion of the gene dapE, encoding the succinyl-diaminopimelate desuccinylase of E. coli, thus disrupting the lysine biosynthesis pathway of E. coli. This deletion was complemented by the plasmid harboring dapL, a gene encoding...
the l,l-diaminopimelate aminotransferase from *Synechocystis* sp. strain PCC 6308 (16).

**Cultivations at the 25-liter scale.** Fermentations were performed under the same parameters that were described by Kroll et al. (16). For cultivations at the 25-liter scale, a Biostat DL30 stainless steel reactor (B. Braun Biotech International, Melsungen, Germany) with a total volume of 42 liters was used. Cultivations were done at 30°C and at a dissolved O2 range of 15 to 100% saturation in the medium; the latter was controlled by varying the agitation rates between 6.8 and 7.0 by controlled addition of 4 N HCl and 25% NH4OH. Foam was removed by a mechanical foam destroyer; if this was not sufficient, the antifoam agent Struktol SB2121 (Schill & Seilacher "Struktol" GmbH, Hamburg, Germany) was added. Dissolved O2, pH, foam, temperature, and optical density (850 nm) were measured during the fermentation, while process control and data processing were done by a digital control unit (DCU) in combination with the MFC/Swin software package (B. Braun Biotech International).

**Cell harvest from 25-liter-scale cultivations.** Cells were harvested by centrifugation at 4°C in a CEPA type Z41 or type Z61 continuous centrifuge (Carl Padberg Zentrifugenbau GmbH, Lahr, Germany). The harvested cells were lyophilized using a Beta 1-16 freeze dryer (Christ, Osterode, Germany), and the cell mass was gravimetrically determined.

**Isolation of CGP.** To isolate CGP from cells cultivated at the 25-liter scale, the lyophilized cells were ground in a ceramic mortar, diluted in demineralized H2O (10 ml/g), and adjusted to a pH of 6.5 by adding concentrated HCl. The solution was stirred at 4°C overnight and then centrifuged for 30 min (4°C; 9,000 × g). The supernatant was neutralized with acetone and drying at 65°C, the soluble CGP was dissolved in 50 mM concentrated HCl. The solution was stirred at 4°C overnight and then incubated at 50°C for 5 h. Then, the entire reaction mixture was passed through an ultrafiltration column (3-kDa exclusion size) to separate the digested dipeptides from the enzyme in the supernatant. A control without CGP ensured that all detected signals derived from the digested CGP.

**RESULTS**

**Synthesis of CGP at the 25-liter scale.** For synthesis of both soluble and insoluble forms of CGP, the *E. coli* dapE-dapL cphA strain was grown at the 25-liter scale in TB medium that contained lactose as an additional carbon source and inducer for the vector system. The cells were harvested after 27 h of cultivation at a cell density of 10.2 g/liter, yielding a total cell dry matter (CDM) of 305 g. The soluble CGP content of the cells was determined to be 25.1% (wt/wt) of the CDM, while another 11.1% (wt/wt) was soluble CGP, resulting in an overall CGP content of 36.2% (wt/wt).
was cooled to room temperature and formed a second layer over the brownish and incubated at 60°C for 12 h. The brownish CGP remained insoluble at this diluted in Tris-HCl (50 mM; pH 7), combined with proteinase K (200 μg/ml), and incubated at 60°C for 12 h. The brownish CGP remained insoluble at this temperature, while the white CGP solubilized. It precipitated after the mixture was cooled to room temperature and formed a second layer over the brownish CGP, which can be seen in the photograph.

First observation of a temperature-dependent separation of soluble and insoluble CGPs from cells of E. coli. Several grams of insoluble CGP were diluted in Tris-HCl (50 mM; pH 7), combined with proteinase K (200 μg/ml), and incubated at 60°C for 12 h. The brownish CGP remained insoluble at this temperature, whereas the supernatant showed no turbidity or coloration. While the soluble CGP contained 36.5 ± 0.5 mol% Lys, the insoluble CGP showed an average Lys content of 16.2 ± 0.9 mol%, Arg. The brownish CGP was composed of 53.4 ± 0.4 mol% Asp, 34.4 ± 0.7 mol% Arg, and 12.2 ± 0.3 mol% Lys, whereas the white CGP was composed of 51.1 ± 0.4 mol% Asp, 20.0 ± 0.2 mol% Arg, and 28.9 ± 0.6 mol% Lys. While there was a slight difference in the Asp contents, the main difference was the 2.4-fold-higher Lys content of the white CGP in comparison to its brownish counterpart.

This raised the question of whether the high Lys content was responsible for the difference in solubility. Since a white color is a good indication of the purity of the CGP, the insolubility of the brownish CGP might also be caused by contaminants left in the polymer sample, which may also be the reason for the slightly increased Asp content of the polymer. However, this can only be the case if these substances are tightly bound to the CGP and therefore cannot be separated from it.

**Separation using a temperature gradient.** For further investigations, three fresh batches of CGP were separated along a temperature gradient from 90°C to 30°C. The CGP was diluted in Tris-HCl buffer (50 mM; pH 7.0), heated to 90°C, and allowed to stand until the unsolubilized CGP had settled at the bottom. The clear supernatant was then withdrawn with a pipette and cooled on ice, which resulted in the precipitation of the previously solubilized CGP. This process was repeated with fresh Tris buffer until no additional CGP was solubilized in the supernatant. This was done to remove solubilized CGP that was trapped between the insoluble CGP particles, since it was not possible to centrifuge the samples at temperatures higher than 40°C.

The fraction of CGP that was extracted at 90°C was then heated to 80°C. At this temperature, less than the total amount of CGP that was soluble at 90°C was again solubilized, and a clear supernatant was separated from the insoluble CGP, as before. This process was repeated in steps of 10°C to a temperature of 30°C. At this temperature, the remaining amount of CGP was too small for an additional step to a lower temperature. The entire separation process showed that not all CGP soluble at a specific temperature is soluble at a lower temperature.

After the temperature separation, an OPA HPLC analysis was performed, which showed a correlation between the solubilization temperature of the CGP and the Lys content. The lower the solubilization temperature of a CGP fraction, the higher the Lys content of the polymer became. The increasing Lys content was paralleled by a corresponding decrease in the Arg content. While the CGP that was not soluble even at 90°C showed only a very low Lys content of about 3.9 ± 0.4 mol% and 43.2 ± 0.3 mol% Arg, the CGP that was soluble at 30°C had an approximately 8-fold-higher content of Lys (31.0 ± 0.5 mol%) and significantly decreased content of Arg (19.0 ± 0.5 mol%). Also noticeable was a slightly increased content of Asp, up to 52.9 ± 0.2 mol%, in the samples with the highest solubilization temperature (80°C to >90°C). The overall changes in the amino acid composition are shown in Fig. 2. Also shown in the figure is the soluble CGP produced by the E. coli dapE-dapL cphA strain, which was separated previously and exhibited an even higher Lys content of 36.5 ± 0.5 mol% and only 13.0 ± 0.0 mol% Arg, thus continuing the overall trend of the separation.

Samples of each CGP were separated by SDS-PAGE to identify possible differences in the molecular masses of the polymer. As Fig. 3 shows, the CGP samples exhibited no significant differences in the average size of the polymer molecules and molecular mass distribution or any tendencies along the temperature gradient.
The average molecular mass (center of the polydisperse area) was estimated to be 24.4 ± 0.6 kDa using the Bio-Rad Quantity One 1-D Analysis Software.

**Investigation of the high Asp content.** The slightly increased Asp content in some samples raised the question of whether all Asp residues of the Asp backbone of the CGP were covalently linked to an Arg or Lys side chain. If this is not the case, the Asp content of the polymer in relation to Arg plus Lys would increase. However, unsubstituted Asp residues should not occur, according to the catalysis mechanism published by Berg et al. (19), and modifications of the Asp backbone have never been detected in vivo and only to a very small degree in vitro (17). Therefore, CGP almost always contains 50 mol% Asp, while the other 50 mol% can consist of a mixture of several compounds in the side chains. Since only samples obtained at the highest solubilization temperature in the gradient seemed to be affected, such modifications might occur during heat treatment. Since unsubstituted Asp residues of the polymer cannot be detected by the standard OPA HPLC protocol, which results in complete hydrolysis of the polymer to its single amino acids, the entire polymer was degraded using the extracellular cyanophycinase CphE from *P. alcaligenes* DIP1. Beside the dipeptides Asp-Arg and Asp-Lys, free Asp residues or Asp-Asp-Arg and Asp-Asp-Lys tripeptides from the backbone could also be separated and thereby detected.

The analysis of the digested polymer showed an increasing content of Asp-Lys dipeptides in CGP fractions solubilized at increasing temperatures, which was paralleled by a simultaneous decrease of Asp-Arg dipeptides (Fig. 4). Furthermore, the analysis of the digestion products showed Asp, as well as Arg, in a molar ratio of approximately 50/50 mol%. Therefore, it is likely that the free amino acids originated from degraded CGP due to the high temperature at the beginning of the gradient.

**DISCUSSION**

Since the first discovery of soluble CGP in *E. coli* in 2002 (4), the reasons for its solubility remained unknown for years. Several theories, including differences in the structure of the polymer; variations in the cell milieu influencing the specificity of the cyanophycin synthetase; or the incorporation of alternative constituents, like Lys, citrulline, and ornithine, have been discussed (6, 8). We are now confident that we have identified one of the factors leading to the formation of soluble CGP in *E. coli*. Our data showed that the Lys content of the CGP corresponds to a solubility change of the polymer itself. The higher the Lys content of the insoluble CGP, the lower the temperature required to solubilize the polymer in aqueous solutions. Therefore, it is obvious that at a certain point the Lys content of the polymer would reach a threshold value above which it would always be present in its soluble form, even at the low temperature during CGP purification, which in our study was about 4°C. For the strain used, this limit is likely to be a Lys content of about 30 mol%. During the separation process, CGP with a higher Lys content would not precipitate at pH 7.0 and would remain in the supernatant, where it needs to be precipitated by the use of EtOH, enabling the isolation of soluble CGP.

Since research was focused on the insoluble CGP for a long time, the presence of the soluble form was often ignored or was most likely not detected or determined. Therefore, soluble CGP was likely discarded during the purification of the insoluble CGP. For example, the *E. coli* dapE-dapL cphA strain used for CGP synthesis in this study, was developed and published in 2011 (16) and reported to be able to synthesize insoluble CGP amounts from 18...
to 25% of the CDM, depending on the medium used for cultivation. Since research on soluble CGP was just starting at that time, no tests on soluble CGP were performed during large-scale cultivations. Recently, we used this strain for the synthesis of insoluble CGP (e.g., in this study), but we also checked for soluble CGP during the purification process. We thereby revealed that while the strain produced a similar amount of insoluble CGP (25.1% of the CDM), it also contained 11.1% soluble CGP with a high Lys content of 37 mol%. This increased the overall CGP content by almost 45% to 36.2% of the CDM, demonstrating the importance of determining the presence of soluble CGP, especially for large-scale production in strains of *E. coli*.

While Frommeyer and Steinbüchel (6) isolated soluble CGP from an *E. coli* strain containing as little as 17% Lys, we achieved a much higher Lys content. The high Lys content of the CGP produced by our strain might be caused by the addiction system used. Since the addiction system interferes with the biosynthesis of Lys, it is likely that, due to the expression of *dapL* on the vector, Lys biosynthesis might be enhanced, leading to an increased pool of Lys in the cytoplasm, which results in a higher incorporation rate.

It is likely that Lys is not the only reason for the formation of soluble CGP, but modifications in the CGP side chains definitely have a large influence on its occurrence. Also, the Lys content at which the CGP is soluble might be different depending on the strain and the cyanophycin synthetase used. In this case, the high Lys content might even mask other factors influencing CGP solubility. Therefore, soluble CGP with less Lys may also occur under certain conditions. Also, the method by which soluble and insoluble CGP are separated during the purification is crucial. The higher the temperature used during separation, the lower the Lys content of the soluble CGP will be, because at higher temperatures, CGP with less Lys is solubilized, as well. This might also explain why the soluble CGP synthesized by Frommeyer and Steinbüchel (6) contained less Lys, since the separation was performed at 20°C compared to 4°C, as performed here.

Another observation was made regarding the general properties of the CGP. As a nonribosomal polypeptide, the polymer chain has no specific size, leading to a mass distribution from 18 to over 30 kDa in recombinant strains, which is especially noticeable on SDS gels (Fig. 3). Our data also showed another characteristic of the CGP. As a nonribosomal polypeptide, the polymer formed from an alternative constituent has led to changes in their solubility and to their separation during purification. Therefore, it is also quite probable that the soluble CGP itself is a mixture of chains with differing amounts of Lys, but only of those with a Lys content high enough to be soluble at a specific point during the purification. Describing soluble and insoluble CGPs as two different polymers would therefore not be appropriate, since both CGP forms derive from the same polymer mixture and are still composed of different polymer chains. In this case, insoluble CGP could be described as a mixture of CGP chains with a Lys content lower than 31 mol%, leading to their insolubility at pH 7.0 and 4°C, while the soluble CGP is a mixture with more than 31 mol% Lys, which is soluble within these parameters.

A possible application for these characteristics is the downstream processing of CGP from large-scale cultivations. The current purification process involves several repeats of solubilizing, precipitating, and washing the CGP (5). This method is time-consuming and does not remove certain contaminants, such as proteins with a similar solubility behavior. To obtain CGP of high purity, an additional digestion with protease K is necessary. Our data showed that extracting the CGP using high temperatures results in highly pure CGP after only a single step of purification, comparable to CGP purified by the established method. Approximately 90% of the synthesized CGP is soluble at 95°C and can be extracted from the supernatant, while proteins are denatured and separated with the insoluble debris. An additional purification step is required to separate soluble and insoluble CGPs. The entire procedure requires only a few hours in comparison to 2 to 3 days using the established method.

Another possible application of the temperature separation is the improvement of CGP dipeptide production. Since many applications for CGP dipeptides require a single type of dipeptide or a specific mixture (9, 10), the separation of undigested CGP by temperature might be suitable to produce CGP with a specific composition or a specific constituent prior to the digestion with CphE. Currently, specific mixtures of CGP dipeptides can only be obtained by mixing already purified dipeptides. Therefore, by separating CGP into a large variety of constituent concentrations, different fractions of CGP can be combined to achieve a desired mixture prior to CGP digestion, which would be far more efficient than combining purified dipeptides.

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**REFERENCES**

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