

Use of Modified BL21(DE3) *Escherichia coli* Cells for High-Level Expression of Recombinant Peanut Allergens Affected by Poor Codon Usage

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We previously cloned a panel of peanut allergens by phage display technology. Examination of the codons used in these sequences indicated that most of the cDNAs contain an excess of the least used codons in *Escherichia coli*, namely AGG/AGA, that correspond to a minor tRNA, the product of the *dnaY* gene. To achieve high-level expression of the peanut allergens, the cDNAs were subcloned into an expression vector of the pET series (Novagen) in order to produce (His)₁₀-tagged fusion proteins in conventional *E. coli* BL21(DE3) cells. The peanut allergens Ara h 1, Ara h 2, and Ara h 6 with an AGG/AGA codon content of 8–10% were only marginally expressed, whereas the peanut profilin Ara h 5, with an AGG/AGA codon content of only 0.8%, was efficiently expressed in these cells. Hence, by using modified BL21(DE3) *E. coli* cells, namely BL21-CodonPlus(DE3)-RIL cells (Stratagene) with extra copies of *E. coli argU*, *ileY*, and *leuW* tRNA genes, it was possible to attain high-level expression of the proteins affected by rare codon usage. IPTG-induced expression of several recombinant peanut allergens, such as Ara h 1, Ara h 2, and Ara h 6, was greatly increased in these special cells compared to the expression yield achieved by conventional *E. coli* hosts. The purification of the soluble and the insoluble fraction of Ara h 2 was performed by metal-affinity chromatography and yielded a total of about 30 mg (His)₁₀-tagged recombinant protein per liter of culture of transformed BL21(DE3)CodonPlus-RIL cells. This is over 100 times more than achieved by production of Ara h 2 in conventional BL21(DE3) cells. © 2000 Academic Press

People who are allergic to peanuts may experience symptoms ranging from mild urticaria, facial swelling,

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and abdominal cramps to hypertension with anaphylactic shock (1,2). The relationship between clinical manifestation and the complex immune responses cannot be investigated without identification and characterization of the allergenic components of peanuts. As previously reported, we isolated a panel of six peanut allergens, Ara h 1, Ara h 2, Ara h 4, Ara h 5, Ara h 6, and Ara h 7 (3), with the application of a recently described technology based on the phage surface display of cDNA expression products (4). Ara h 5 with an estimated molecular weight of 14 kDa corresponds to the well-known plant allergen profilin (5). The other five peanut allergens with molecular weights of 14.5 to over 63 kDa show significant sequence similarities to the family of seed storage proteins.

In the present paper, we focus on the expression of four of the identified allergens, necessary for further characterization experiments and also for improvement of diagnostic and immunotherapeutic approaches. The cDNAs were subcloned into the expression vector pET-16b (Novagen) that uses the T7 RNA polymerase-responsive promoter (6) to produce (His)₁₀-tagged fusion proteins. This resulted in an ineffective expression of Ara h 1, Ara h 2, and Ara h 6 in conventional BL21(DE3) *Escherichia coli* cells on the one hand and an efficient expression of Ara h 5 on the other hand. Closer examination of the codons used within the four sequences indicated that Ara h 1, Ara h 2, and Ara h 6 contain 8–10% of the least used arginine codons AGG/AGA in *E. coli* whereas Ara h 5 contains only 0.8% of these rare *E. coli* codons.

Although *E. coli* has a remarkable capacity to produce large quantities of protein, there are limits when the codon usage in the mRNA of the recombinant gene differs from the *E. coli* host cells (7–9). Within *E. coli*, a clear bias exists among the 61 amino acid codons found within the population of mRNA molecules, and the level of cognate tRNA appears to be directly pro-

portional to the frequency of codon usage (10–12). A complete compilation of codon usage of the sequences placed in the GenBank database can be found at <http://www.kazusa.or.jp/codon/> (13). A subset of codons, namely AGG/AGA (arginine), AUA (isoleucine), CUA (leucine), CGA (arginine), and CCC (proline), are the least used codons in *E. coli* that correspond to rare tRNAs. Recent studies suggest that an excess of any of these codons creates problems during translation, leading to a reduction in quantity of the protein synthesized (14–16). Errors are detectable as frameshifts and decreased levels of expression. More subtle changes, such as mistranslation, may also occur (17).

Referring to these facts, the translational problems with the mRNA of some of the recombinant peanut allergens could be explained by the excess of low-abundance tRNA codons within these sequences. Hence, to achieve high-level expression of these peanut allergens, we used the recently generated BL21(DE3)CodonPlus-RIL cells (Stratagene) carrying extra copies of the argU, ileY, and leuW tRNA genes (18). In this study we could show that the production of the peanut allergens Ara h 1, Ara h 2, and Ara h 6 with an AGG/AGA content of 8–10% were enhanced significantly with these modified cells coexpressing the cognate tRNA genes compared to the expression rates within conventional BL21(DE3) cells. Samples were analyzed on SDS–PAGE and visualized by staining with Coomassie brilliant blue. Proteins were transferred to a nitrocellulose membrane for Western blot experiments. The specific binding of the patient's IgE to the recombinant allergens were detected. In addition, we report the purification of the (His)₁₀-tagged recombinant Ara h 2 by Ni–NTA² metal-affinity chromatography under native and under denaturing conditions (19).

MATERIALS AND METHODS

Expression of Recombinant Peanut Allergens

For high-level expression, the coding region of the four different cDNAs of Ara h 1, Ara h 2, Ara h 5, and Ara h 6 were cloned in frame into the T7 promotor-driven expression vector pET-16b (Novagen, Madison, WI). This vector contains an N-terminal (His)₁₀-tag, which can be used as affinity ligand for purification purposes. The recombinant plasmids were used to transform into electrocompetent *E. coli* BL21(DE3) cells (Novagen) or into BL21(DE3)CodonPlus-RIL cells (Stratagene) using an electroporation apparatus (EasyJect Optima, peqlab, Erlangen, Germany). A 10-ml culture of each expression, in LB broth supplemented with 50 µg/ml carbenicillin, was induced by addition of 1 mM β-D-thiogalactopyranoside (IPTG) at $A_{600\text{nm}} = 0.7$. Then incubation continued at three dif-

ferent temperatures (22, 30, 37°C). Of the total *E. coli* lysates, 1 ml each was harvested after 2, 4, and 20 h by centrifugation (4000 rpm, 10 min) and then resuspended in 100 µl of reducing sample buffer (20 mM Tris, pH 6.7, 2 mM EDTA, 2% (w/v) SDS, 25% (v/v) glycerin, 3% (v/v) β-mercaptoethanol, bromophenol blue). After SDS–PAGE analysis of 5 µl of each sample, using 5% stacking gels and homogenous 15% separating gels (20), proteins were visualized by staining with Coomassie brilliant blue R-250 (Promega/Serva). For IgE immunoblot analysis separated proteins were transferred to nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany) using a semidry device (21). After blocking, patient serum diluted 1:50 in 0.1M Tris-buffered saline (pH 7.4) was used as first antibody. The specific binding of the patient's IgE to the recombinant proteins was detected by incubating the membrane for 2 h with alkaline phosphatase-conjugated anti-human IgE (Allergopharma, Reinbek, Germany) as second antibody. Bands were visualized with nitroblue tetrazolium chloride (Merck) including 5-bromo-4-chloro-3-indolyl phosphate potassium salt (Promega/Serva).

Purification of Recombinant (His)₁₀ Ara h 2

The soluble fraction of the recombinant Ara h 2 was purified by Ni–NTA metal-affinity chromatography under nondenaturing conditions. In brief, a 1 liter culture of transformed BL21-CodonPlus (DE3)-RIL cells carrying the pET16b-Ara h 2 plasmid was grown at 37°C in LB medium containing 50 µg/ml carbenicillin to a cell density of $A_{600\text{nm}} = 0.7$. Expression was induced by adding 1 mM IPTG and incubation continued at 30°C for 3 h. The cell pellet was collected (4000 rpm, 20 min), dissolved in 30 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9), and disrupted with a French pressure cell disruption apparatus (SLM Aminco, Spectronic Unicam Instruments Inc., Cambridge, UK). Insoluble material of the cell lysate was removed by centrifugation (10,000 rpm, 30 min) and stored at –20°C until further purification of the inclusion bodies under denaturing conditions. The supernatant of the disrupted cells was bound to 5 ml Ni–NTA Superflow resin (Qiagen, Hilden, Germany) in a Falcon tube for 1 h at RT with gentle shaking. The resin was loaded into a column (Pharmacia) for FPLC purification. After the column had been washed with 30 ml binding buffer and 30 ml wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9), respectively, the (His)₁₀-tagged recombinant Ara h 2 was eluted with 50 ml elution buffer (250 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9). The purified protein within the pooled column eluate was desalted by gel filtration using PD-10 Sephadex G-25 M columns (Pharmacia).

² Abbreviations used: Ni–NTA, nickel–nitriloacetic acid; IPTG, β-D-thiogalactopyranoside; RT, room temperature.

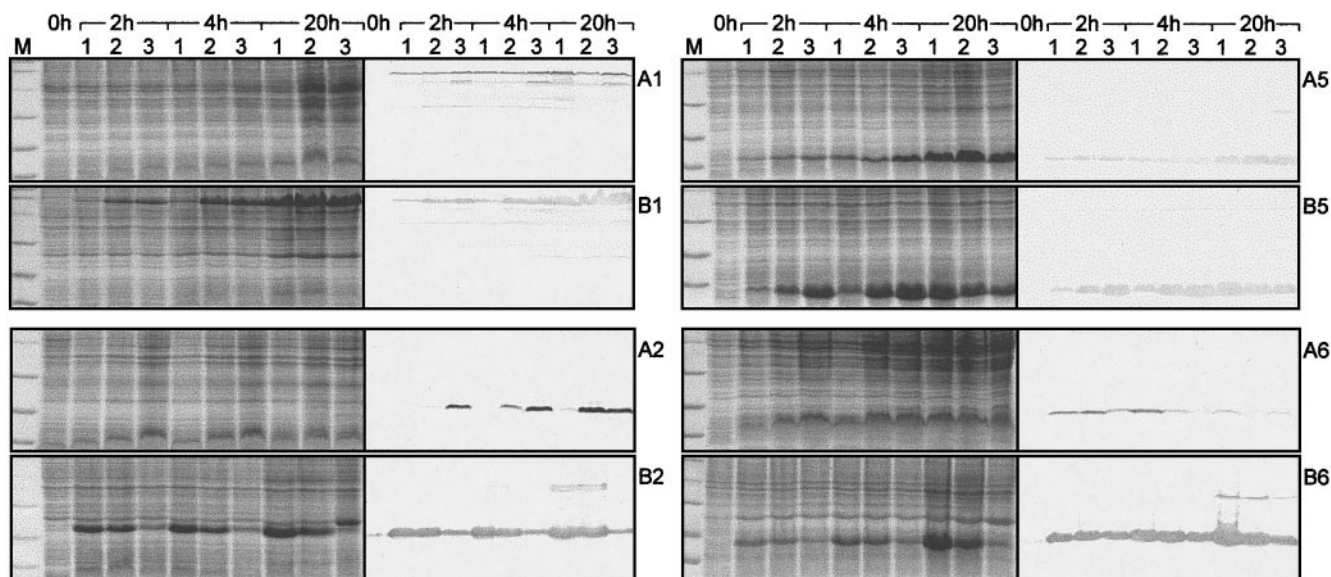


FIG. 1. Comparison of expression yields of the recombinant His-tagged peanut allergens Ara h 1, Ara h 2, Ara h 5, and Ara h 6 with conventional BL21(DE3) cells (A1, A2, A5, A6) and with modified BL21(DE3)CodonPlus-RIL cells (B1, B2, B5, B6). Expression was performed in a 10-ml culture over a period of 20 h at 22°C (lane 1), 30°C (lane 2), and 37°C (lane 3) after induction. Aliquots of the total cell lysates were collected at 0, 2, 4, and 20 h after IPTG induction, dissolved in sample buffer containing 2-mercaptoethanol, and analyzed by 15% SDS-PAGE. The samples were visualized by Coomassie blue staining (left) and by Western blot analysis (right) with a serum of a peanut allergic used as first antibody in order to show IgE-binding capacity of the recombinant proteins. Lane M, markers, molecular mass 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa from top to bottom.

Purification of the Ara h 2 highly enriched in the inclusion bodies was performed under denaturing conditions with 8 M urea buffers. The cell pellet was washed with 10 ml 1 M urea buffer (1 M urea, 0.1 M NaH_2PO_4 , 0.01 M Tris-HCl, pH 8.0) and after a centrifugation step (10,000 rpm, 30 min), the pellet was resuspended in order to solubilize inclusion bodies with 10 ml lysis buffer (8 M urea, 0.1 M NaH_2PO_4 , 0.01 M Tris-HCl, pH 8.0). After a further centrifugation (10,000 rpm, 30 min), the supernatant was bound to 5 ml Ni-NTA resin as described for the nondenaturing purification. The resin was loaded into a column for batch purification. After the column had been washed with 50 ml lysis buffer and 40 ml wash buffer (8 M urea, 0.1 M NaH_2PO_4 , 0.01 M Tris-HCl, pH 6.3), respectively, elution of the (His)₁₀-tagged recombinant Ara h 2 was carried out using 40 ml elution buffer (8 M urea, 0.1 M NaH_2PO_4 , 0.01 M Tris-HCl, pH 4.5). In order to refold the solubilized denatured Ara h 2, the pooled column eluates were dialyzed for 4 days: twice against 4 liters of 5 mM cysteine for 24 h and twice against 4 liters of H_2O for 24 h, respectively. Lyophilized samples were stored at -20°C.

Bound, washed, and eluted fractions of both purification procedures were assessed by 15% Coomassie-stained SDS-PAGE. Protein concentrations of each of the pooled purification fractions and of the desalted solutions after the gel filtration or dialysis procedure were determined by absorbance at 280 nm and by

using a Coomassie protein assay reagent (Pierce, Rockford, IL).

RESULTS AND DISCUSSION

Although the BL21(DE3) *E. coli* strain is a convenient and effective host for protein expression, the production of three of four recombinant peanut allergens subcloned into the pET-16b vector was limited (Fig. 1, A1, A2, A6) with these cells. Only the peanut allergen Ara h 5 with a 396-bp cDNA-coding region and a predicted MW of 14 kDa was successfully expressed in these conventional *E. coli* cells (Fig. 1, A5). Examination and comparison of the codon usage of these four peanut allergen sequences indicated that the Ara h 5 mRNA and *E. coli* mRNAs have a similar codon usage, whereas the codon usage of the other three mRNAs of Ara h 1, Ara h 2, and Ara h 6 is very different from that of highly expressed homologous genes in *E. coli*. This results in the presence of many rare *E. coli* codons in the sequences encoding the three peanut allergens. They contain an anomalously high frequency of the rare arginine codons AGG and AGA when compared to genes of *E. coli*. These are the least frequent codons in genes of *E. coli* and are recognized by the rarest tRNAs. *E. coli* only uses three to four AGG/AGA codons per 1000 codons (13) whereas the peanut allergens Ara h 1, Ara h 2, and Ara h 6 contain 54, 96, and 81 AGG/AGA codons, respectively, per 1000 codons (Table 1). The

TABLE 1
Characteristics of Four cDNAs Coding for Peanut Allergens

Allergen ^a	GenBank Accession No.	Protein family	cDNA/coding region (bp)	ORF	MW ^b (kDa)	aa residues	Codon usage AGG/AGA ^c (%)	AUA ^d (%)	CUA ^e (%)
Ara h 1	L34402	Vicilin	1949/1611	237–1848	63.2	536	5.41	0.75	0.00
Ara h 2	L77197	Conglutin	717/474	2–475	18.6	157	9.55	0.64	0.64
Ara h 5	AF059616	Profilin	743/396	17–412	14.0	131	0.76	0.00	0.00
Ara h 6	AF092846	Conglutin	627/375	16–390	14.5	124	8.06	2.42	0.00

Note. MW, molecular weight; aa, amino acid.

^a Nomenclature officially approved by the WHO/IUIS Allergen Nomenclature Sub-Committee (30).

^b Values calculated from the deduced amino acid sequences without His-tag.

^{c,d,e} Codon frequency of *E. coli* mRNAs: AGG/AGA, 0.43%; AUA, 0.53%; CUA, 0.4% (13).

minor tRNA^{Arg(AGG/AGA)} has been shown to be a limiting factor in the bacterial expression of several genes (10–12). The presence of rare codons reduces the translational rate (14–16) and, depending on the context, this can strongly modulate translational efficiency (22). These observations imply that heterologous genes, enriched with codons that are rarely used by *E. coli*, may not be expressed efficiently in *E. coli* cells. Referring to these facts, the translational problems with the mRNAs of the three peanut allergens Ara h 1, Ara h 2, and Ara h 6 were to be expected.

Until recently, three strategies were usually recommended to solve this problem (23): first, use a host containing a plasmid with the appropriate tRNA (10,24,25); second, synthesize the gene to replace rare codons with frequently used codons (26); or third, use a eukaryotic expression system. Today, the fourth alternative is the use of *E. coli* hosts that carry extra copies of the argU tRNA gene. Just recently, such cells were generated by Stratagene (18). To overcome translational problems apparently associated with the presence of rare arginine codons in three of four peanut allergens, we used the *E. coli* host BL21(DE3)CodonPlus-RIL for the expression studies of the four peanut allergens. We could demonstrate the efficiency of these modified cells to facilitate protein production in genes affected by poor codon usage. Apart from the additional tRNA^{Arg(AGG/AGA)} genes, these modified BL21(DE3) *E. coli* cells carry extra copies of tRNA^{Ile(AUA)} and tRNA^{Leu(CUA)} genes coding for tRNAs that recognize rare isoleucine and leucine codons, respectively. The frequency of the AUA and CUA codons within the four peanut allergens is comparable to the frequency within *E. coli* mRNAs. Only Ara h 6 has a slightly higher AUA content than *E. coli* mRNAs with 2.4% compared to 0.53% (Table 1). Therefore, the favorable effect of these cells on the peanut allergen production seems to be predominantly due to the extra argU tRNA gene copies.

Ara h 1 (GenBank Accession No. L34402) with a predicted MW of around 63 kDa (without (His)₁₀-tag) and an AGG/AGA content of 5.4%, Ara h 2 (GenBank

Accession No. L77197) with a MW of around 19 kDa and an AGG/AGA codon usage frequency of 9.6% and Ara h 6 (GenBank Accession No. AF092846, 14.5 kDa, 8.1% AGG/AGA content) were only marginally expressed in conventional BL21(DE3) cells. Figure 1 (A1, A2, A6) demonstrates that on the Coomassie blue-stained SDS-PAGE no additional protein band of the appropriate molecular weights appeared after IPTG induction, regardless whether the incubation after induction at three different temperatures continued for 2, 4, or 20 h. Only by Western immunoblotting experiments a very low amount of IgE-binding recombinant protein became visible. It is noticeable that the expression of Ara h 1 is accompanied by truncated translation products as also shown by other authors (27). Compared to these expression studies, the production of three of the four peanut allergens, affected by rare codon usage, greatly increased in the special BL21(DE3)CodonPlus-RIL cells as also shown by SDS-PAGE analysis and subsequent Coomassie blue staining (Fig. 1, B1, B2, B6). For the four peanut allergens, maximal expression yields within this modified *E. coli* host were obtained at different temperatures with overnight cell growth after IPTG induction. The IgE-binding capacity of the recombinant proteins was confirmed by Western blot analysis (Fig. 1).

Figure 1 (A5, B5) demonstrates that the production efficiency of Ara h 5, showing no excess of rare codon usage, is similar within the two *E. coli* strains. The Ara h 5 protein expressed in the modified BL21(DE3)CodonPlus-RIL cells shows exactly the predicted molecular weight of 14 kDa. This protein is expressed without (His)₁₀-tag in an expression vector of the pET series (Novagen) without any fusion tag. All the other cDNAs shown in this study are produced as fusion proteins subcloned into the pET-16b vector with an N-terminal (His)₁₀-tag for purification purposes. In contrast to the other three allergens, corresponding to plant storage proteins, profilins do not need a fusion tag for purification because they

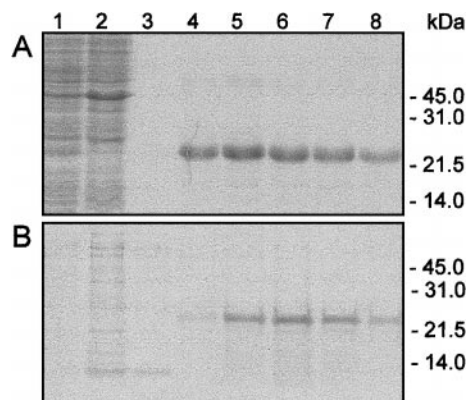


FIG. 2. Purification of (His)₁₀-tagged recombinant Ara h 2. Portions of each of the purification fractions were separated on a 15% SDS-PAGE and stained with Coomassie blue. (A) Batch purification of recombinant (His)₁₀-Ara h 2 under denaturing conditions with Ni-NTA resin and 8 M urea buffers. Lane 1, column eluate following the washing step with 8 M urea buffer, pH 8.0 (flowthrough); lanes 2 and 3, column eluates following the washing step with 8 M urea buffer, pH 6.3; lanes 4–8, column eluates following elution of the (His)₁₀-tagged recombinant Ara h 2 with 8 M urea buffer, pH 4.5. (B) FPLC purification of recombinant (His)₁₀-Ara h 2 with Ni-NTA Superflow resin under native conditions with imidazole buffers. Lanes 1–3, column eluates following the 60 mM imidazole buffer washing step; lanes 4–8, column eluates following elution of the (His)₁₀-tagged recombinant Ara h 2 with 250 mM imidazole buffer.

can be efficiently purified by affinity interaction with poly-L-proline (28). The detailed purification procedure of the peanut profilin performing affinity chromatography with PLP–Sephacrose was described previously by our group (29). The expression yield amounted to 30 mg/liter culture of highly pure recombinant allergen in accordance with the expression yield of (His)₁₀-tagged Ara h 5 in conjunction with conventional BL21 cells, purified by metal-affinity chromatography.

Unlike peanut profilin (Ara h 5), which is totally expressed in the soluble fraction, Ara h 2 was located both in the soluble fraction and in the insoluble frac-

tion, suggesting that Ara h 2 is partly enriched and aggregated in inclusion bodies in the cytoplasm of *E. coli*. The two fractions were purified separately following harvest of the induced cells at $A_{600\text{nm}} = 1.5$ with a wet weight of 8.2 g, cell disruption with a French pressure apparatus, and a subsequent centrifugation. The soluble fraction was purified by Ni-NTA metal-affinity chromatography under native conditions with imidazole using an FPLC apparatus (Fig. 2B). The total yield of the eluted recombinant protein was estimated as 4.5 mg/liter culture (Table 2). Purification of Ara h 2 from solubilized inclusion bodies was performed under denaturing conditions using 8 M urea (Fig. 2A) with a final yield of column eluted recombinant protein of about 24 mg/liter culture (Table 2). Figure 2 shows the Coomassie-stained SDS-PAGE with aliquots of the fractions of the various steps of both purification procedures. Pooling of the elution fractions from the Ni-NTA affinity chromatography of the soluble fraction as well as of the insoluble fraction yielded highly pure (>95% purity) recombinant (His)₁₀-Ara h 2 with a total yield of approximately 28.5 mg/liter culture.

Concerning Ara h 2, containing seven cysteine residues, the isolated inclusion body proteins usually contain a certain amount of interchain disulfide bonds which reduces the solubility of the proteins in the absence of reducing agents such as glutathione, cysteine, or 2-mercaptoethanol (31). Therefore, we performed the dialysis procedure with 5 mM cysteine solution in order to have a favorable effect on renaturation with a minimal loss of purified protein of less than 10% (Table 2).

In summary, in the present study, we describe the successful bacterial expression of heterologous proteins that are difficult to express in conventional *E. coli* hosts. By using special *E. coli* cells with extra copies of argU tRNA genes, we attained high-level expression of three peanut allergens, Ara h 1, Ara h 2, and Ara h 6, affected by poor codon usage. IPTG-induced expression

TABLE 2
Purification of (His)₁₀-Ara h 2

Fraction		Protein (mg/liter)	Percentage of total protein (mg/liter)
Soluble fraction	Supernatant after French press of the first pellet ^a with 5 mM imidazole buffer	830.0 ^b	100
	Column eluates after Ni-NTA affinity chromatography	4.5 ^c	0.54
	Eluate after use of the PD-10–Sephadex column	4.0 ^{b,c}	0.48
Inclusion bodies	Supernatant after lysis of the second pellet with 8 M urea buffer	214.5 ^c	100
	Column eluates after Ni-NTA affinity chromatography	24.0 ^c	11.2
	Eluate after dialysis	22.0 ^{b,c}	10.3

Note. Protein concentrations are based on a 1-liter culture (8.2 g wet wt)^a and were determined by using the Coomassie protein assay reagent (Pierce)^b and/or by measuring $A_{280\text{nm}}$ ^c applying the following formula: $A_{280\text{nm}}/\epsilon = \text{mg protein/ml}$; $\epsilon = [(5600 \times \text{Trp}) + (1320 \times \text{Tyr})]/\text{MW}$; $\epsilon_{\text{Ara h 2}} = 0.705$.

of these three genes were over 100-fold increased in BL21(DE3)CodonPlus-RIL cells compared to the expression yield achieved by conventional *E. coli* cells like BL21(DE3) cells.

The purification of the expressed proteins could be successfully accomplished by metal-affinity chromatography, illustrated by the purification of the soluble as well as of the insoluble fraction of Ara h 2. Compared to 225 µg/liter culture achieved by Ara h 2 expression in conventional *E. coli* hosts, a total amount of about 30 mg of purified (His)₁₀-tagged recombinant Ara h 2 (column eluate) per liter of culture of transformed BL21(DE3)CodonPlus-RIL cells was obtained.

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