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Mutagenesis of the L-Amino Acid Ligase RizA Increased the Production of Bioactive Dipeptides

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Abstract: The L-amino acid ligase RizA from *B. subtilis* selectively synthesizes dipeptides containing an N-terminal arginine. Many arginyl dipeptides have salt-taste enhancing properties while Arg-Phe has been found to have an antihypertensive effect. A total of 21 RizA variants were created by site-directed mutagenesis of eight amino acids in the substrate binding pocket. The variants were recombinantly produced in *E. coli* and purified by affinity chromatography. Biocatalytic reactions were set up with arginine and four amino acids differing in size and polarity (aspartic acid, serine, alanine, and phenylalanine) and were analyzed by RP-HPLC with fluorescence detection. Variant T81F significantly improved the yield in comparison to wild type RizA for aspartic acid (7 to 17%), serine (33 to 47%) and alanine (12 to 17%). S84F increased product yield similarly for aspartic acid (7 to 17%) and serine (33 to 42%). D376E increased the yield with alanine (12 to 19%) and phenylalanine (11 to 26%). The largest change was observed for S156A, which showed a yield for Arg-Phe of 40% corresponding to a 270% increase in product concentration. This study expands the knowledge about positions governing the substrate specificity of RizA and may help to inform future protein engineering endeavors.



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1. Introduction

Dipeptides constitute a promising substance class and are often more than the sum of their parts. They have physical properties different from their corresponding amino acids like improved heat stability or solubility [1] and may induce pharmacological or other bioactive effects [2]. Several dipeptides have been described as antihypertensives [3,4] while others have been implicated in possible medical applications due to their anticancer [5] or neuroprotective properties [6–9]. The greatest industrial importance belongs to the low-calorie sweetener aspartame, the methyl ester of Asp-Phe, which currently has an estimated global market of 25,000 tons per year [2,10]. Additionally, dipeptides often have effects on other tastes and can possess salt-taste [11–13] or umami-enhancing [14,15] properties.

The chemical synthesis of dipeptides is a multi-step process necessitating the use of protecting groups, which increases waste and complicates purification [2,16] Biocatalysis has emerged as a more sustainable route for performing highly specific reactions without the need for protecting groups or harmful reagents [17,18]. In the past, identifying an enzyme with the fitting natural activity for the desired purpose was a laborious task; today an abundance of bioinformatic tools exists to both facilitate the discovery of new enzymes and to adapt existing ones to the desired bioprocess by protein engineering [19]. A recent development in biocatalytic dipeptide synthesis was the discovery of L-amino acid ligases (LALs; EC 6.3.2.28), which synthesize dipeptides from their respective amino acids using ATP (adenosine triphosphate). The discovery of the first LAL YwfE (also called BacD) from *Bacillus subtilis* was published in 2005 [20]. It synthesized a large

variety of different dipeptides and generally preferred smaller amino acids like alanine as the N-terminal and larger amino acids like phenylalanine as its C-terminal substrate. It was also the first LAL, for which a structure was published [21]. Another LAL was identified in *Ralstonia solanacearum* (RsLAL) with substrate specificities for N- and C-terminal substrates roughly opposite to those of BacD [22]. The structure for the LAL from *Bacillus licheniformis* NBRC12200 (BI-LAL) enabled the structural comparison between LALs with different substrate specificities and thus gave insight into how these differences relate to the structural level [21,23]. Later, this was used in the engineering of BI-LAL for the production of Met-Gly [12]. Another recent engineering study improved the production of Pro-Gly by the LAL TabS from *Pseudomonas syringae* NBRC14081 [24]. Among these enzymes with different substrate specificities, RizA from *Bacillus subtilis* NBRC3134 stands out due to its high N-terminal specificity accepting only arginine, but relaxed C-terminal specificity accepting every amino acid except proline [25].

We previously worked on the production of the salt-taste enhancing dipeptides Arg-Ser, Arg-Ala, and Arg-Gly with RizA coupled to acetate kinase for ATP regeneration to remove the cost of ATP as the major economic obstacle of this reaction [26]. Apart from these salt-taste enhancing dipeptides, RizA should also be capable of producing Arg-Phe, which has been described as antihypertensive [4,27]. However, preliminary tests showed very low yields. Since a crystallographic structure of RizA has been published [28], a mutagenesis study of the substrate binding pocket of RizA was conceived to increase the production of several bioactive dipeptides and to gain knowledge about the substrate specificity of LALs for future engineering endeavors.

2. Results and Discussion

2.1. Selection of Mutations

The selection of important positions for substrate specificity was based on the structure of RizA, a sequence alignment of RizA with BacD, BI-LAL [23], RsLAL [22], and TabS [27], and data from the literature on the substrate specificity and engineering of these LALs. Since the published structure of RizA (pdb: 4wd3) does not contain a substrate, a structural alignment with BacD containing a substrate analog of the Ala-Phe dipeptide (pdb: 3vmm) was used to approximate substrate binding as has been previously done in the original publication on the RizA structure [28] and also for the engineering of BI-LAL [12].

To gain insight into the substrate specificity of RizA, eight different amino acids in the substrate binding pocket were chosen (Figure 1). Threonine 81 (T81), lysine 83 (K83), serine 84 (S84), serine 156 (S156) and alanine 158 (A158) were selected in the C-terminal binding pocket. Additionally, glycine 289 (G289), isoleucine 291 (I291) and aspartic acid 376 (D376) were chosen in the N-terminal binding pocket.

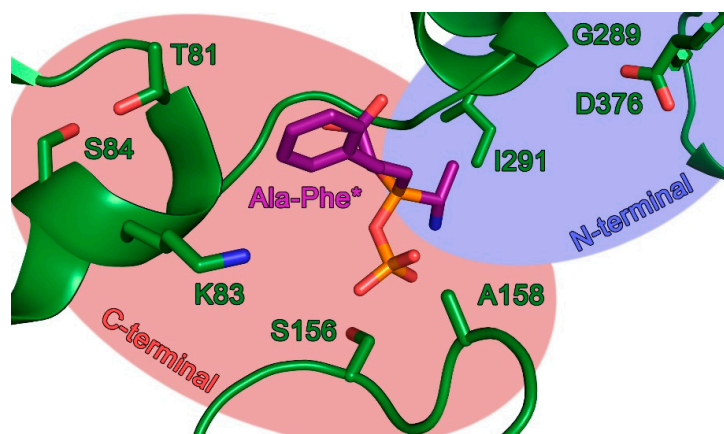


Figure 1. Mutated residues of RizA (green) in the C-terminal (red) and N-terminal (blue) binding pocket (PDB ID: 4wd3). The structure contains the phosphorylated phosphino-analog of L-Ala-L-Phe (purple) from the structural alignment with BacD (PDB ID: 3vmm). Structures were displayed using PyMOL [29].

T81, K83, and S84 are located on an α -helix very close to the C-terminal substrate (Figure 2a). In between them is E82, which is strictly conserved among LALs (Figure 2b) and has been implicated as an essential residue for the catalytic cycle [21,30]. Mutation of the homologous E109 to alanine in BacD resulted in a nearly total loss of activity. In contrast, the neighboring residues differ greatly depending on the LAL, and their close proximity to the substrate suggests a role in substrate specificity. K83 has already been recognized for this function and mutations of the homologous P85 in BI-LAL [12], S85 in TabS [24] and L110 in BaLAL [31] were used for successfully tuning the substrate specificity. Because of its apparent importance, six variants were constructed. K83L/P/S/F were chosen because of their presence in the other LALs (Figure 2b). Additionally, K83R was chosen because of the similar charge to lysine but larger size, and K85T since S85T improved activity in TabS [24]. Position 81 has been implicated as N108 in BacD in substrate binding, but the only engineering attempt was made in BaLAL, where N108F improved product formation [31]. Here, the homologous amino acids in other LALs were chosen for position 81 with the addition of alanine. S84 has—to our knowledge—never been targeted for its effect on substrate specificity, perhaps because of its greater distance from the substrate. However, due to its position and likely interaction with T81 (Figure 2a), an influence would still be plausible and S84F/L were created.

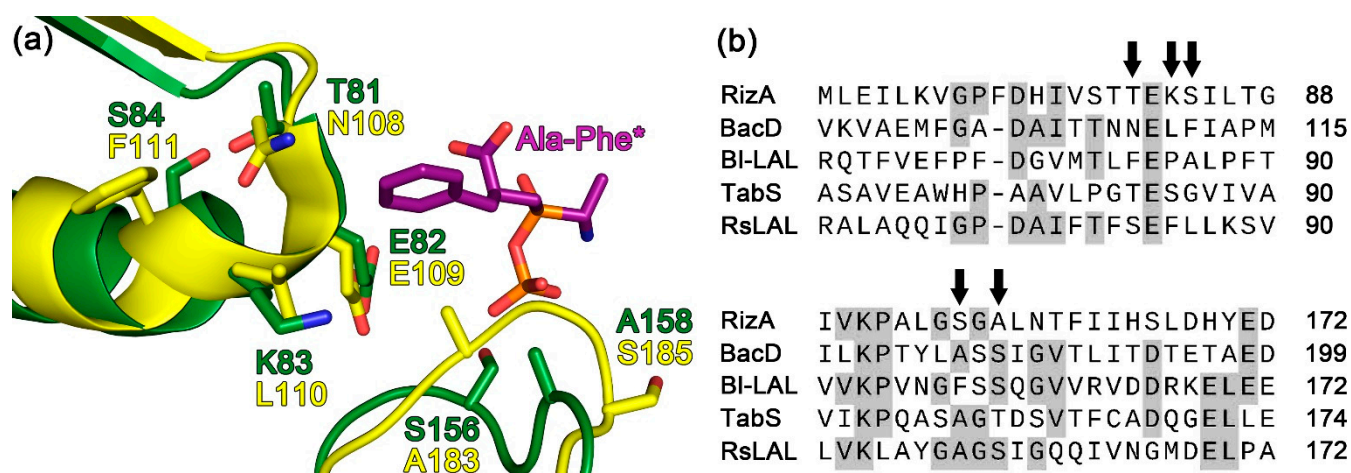


Figure 2. Residues in the C-terminal binding pocket. (a) Structural alignment of RizA (green; PDB ID: 4wd3) with BacD (yellow; PDB ID: 3vmm) containing Ala-Phe analog (purple). The structures were displayed using PyMOL [29]. (b) Sequence alignment of RizA with other LALs. Arrows indicate positions that were mutated in RizA. Grey highlighting indicates conservation above 50%.

S156 and A158 are located on a different loop at the side of the phosphorylated substrate (Figure 2a). In contrast to positions 81 to 84, the structural alignment with the homologous loop in BacD showed large differences in this part of the enzymes. While A158 in RizA is oriented towards the C-terminal binding pocket, S185 of BacD is oriented further towards the N-terminal binding pocket. Besides their proximity to the substrate, both positions were also noticeable on the sequence level. Position S156 was relatively conserved and contained an alanine in all other LALs except BI-LAL, while at A158 all LALs contained a serine or the similar threonine. As a result, S156A/F and A158S/T were chosen for mutagenesis.

In the N-terminal binding pocket, G289 and I291 are located at the beginning of an α -helix (Figure 3a). The loop before the α -helix is located directly at the N-terminal substrate and is highly conserved (Figure 3b) containing, among others, R285, whose homologous R328 in BacD has been identified as an essential residue for catalysis involved in forming an oxyanion-hole for the phosphorylated intermediate [32]. In the same enzyme, both W332 and M334 corresponding to G289 and I291 in RizA (Figure 3a,b) have been strongly associated with N-terminal substrate specificity and the role of W332 was corroborated both

by mutagenesis analysis and crystallographic study of W332A. The position equivalent to I291V was targeted in TabS by saturation mutagenesis, and H294D was part of a double mutant with increased production of Pro-Gly [24]. D376 was proposed as important for binding arginine as the N-terminal substrate and an explanation for the high specificity of RizA for this amino acid [28]. Because of their main role in N-terminal specificity, only few mutations of these residues were examined for their role: G289S, I291V, and D376E.

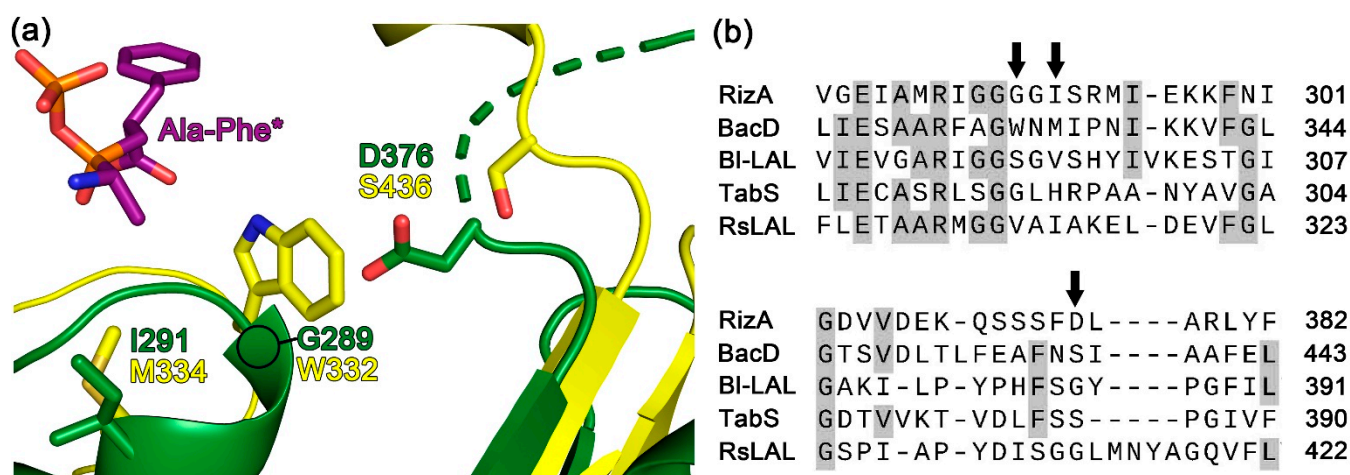


Figure 3. Residues in the N-terminal binding pocket. (a) Structural alignment of RizA (green; PDB ID: 4wd3) with BacD (yellow; PDB ID: 3vmm) containing Ala-Phe analog (purple). The structures were displayed using PyMOL [29]. (b) Sequence alignment of RizA with other LALs. Arrows indicate positions that were mutated in RizA. Grey highlighting indicates conservation above 50%.

2.2. Biocatalytic Performance of RizA Variants with Different Substrates

2.2.1. Recombinant Production of RizA Variants and Biocatalysis Setup

The desired mutations were introduced by site-directed mutagenesis. The variants were recombinantly produced in *E. coli* BL21 (DE3) and purified by affinity chromatography. A panel of five amino acids was chosen representing a spectrum of different sizes, polarities, and properties of the resulting dipeptides. All reactions contained arginine as the N-terminal amino acid, and reactions containing only arginine were performed to compare the formation of Arg-Arg in these reactions with those containing a second amino acid and Arg-Arg as a side product. Moreover, it represented a large, basic amino acid. In contrast, aspartic acid is a small, acidic amino acid and the resulting Arg-Asp a moderate salt-taste enhancer. Serine and alanine are both small amino acids differing in polarity and were also chosen, since both resulting heterodipeptides are strong salt-taste enhancers [25]. Finally, phenylalanine was included as a large, non-polar amino acid and since Arg-Phe has been described as antihypertensive [4]. Biocatalysis was performed using ATP regeneration and dipeptide monitoring by RP-HPLC with fluorescence detection as previously described [26]. Based on these results, a reaction time of 20 h was chosen because the yield with wild type RizA approached equilibrium at that time. The data was first evaluated separately for each amino acid combination in terms of main product (Arg-X) formation and the specificity regarding the formation of the side product (Arg-Arg). At last, the effect of the mutations on the biocatalytic performance was analyzed to increase the understanding of the substrate specificity of LALs and provide possible targets for engineering.

2.2.2. Arginine Only

With arginine being the only substrate, wild type RizA produced 5.6 mM Arg-Arg corresponding to a yield of 19% (Figure 4). The highest concentrations were found in the samples from K83F and S156F containing 10.2 and 10.5 mM Arg-Arg, respectively. Many mutations in the C-terminal binding pocket increased the formation of Arg-Arg, while mutations in the N-terminal binding decreased it with the exception of D376E. S84F

performed similarly to the wild type, while S84L was among the variants containing the least Arg-Arg and constituted the only mutation in the C-terminal binding pocket that reduced Arg-Arg formation.

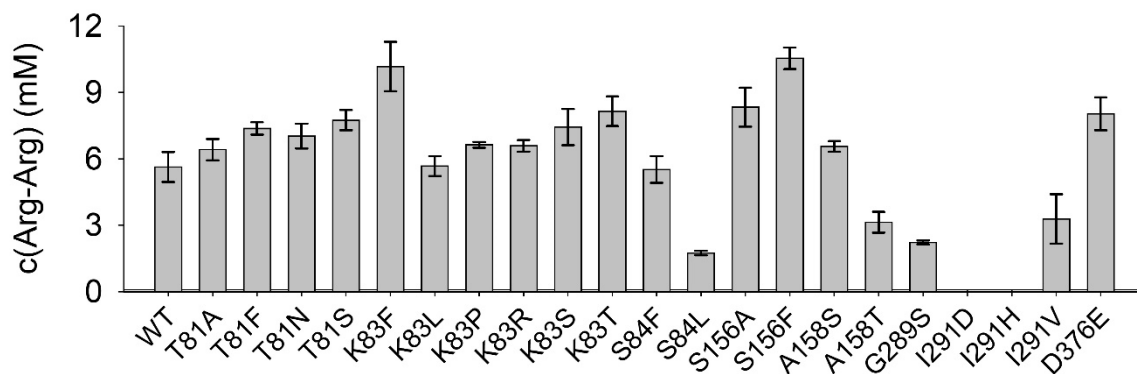


Figure 4. Formation of Arg-Arg by the RizA variants. 60 mM arginine and 20 h reaction time at 25 °C. Reactions were set up in triplicates and data is presented as mean \pm standard deviation.

2.2.3. Arginine + Aspartic Acid

With aspartic acid, the wild type enzyme produced only 2.0 mM Arg-Asp (7% yield), which was the lowest yield among the examined heterodipeptides (Figure 5). In comparison, both T81F and S84F increased Arg-Asp formation to 5 mM. Most of the mutations in the C-terminal binding pocket led to product concentrations comparable to the wild type or above while both S156A/F alongside the C-terminal binding pocket mutations decreased the Arg-Asp concentration below 1 mM. To evaluate the formation of the side product Arg-Arg versus the main product Arg-Asp, the specificity ratio Arg-Asp/Arg-Arg was calculated. (Figure 5). Most variants showed ratios between 2.0 and 1.0. In general, the change in ratio correlated with the increase in Arg-Asp formation and increased from 1.3 in the wild type reaction to 2.8 and 2.4 in the variants with the highest heterodipeptide increase, T81F and S84F. In line with this, the variants with the lowest Arg-Asp formation also showed the lowest ratios. In contrast, the specificity in the K83 variants remained on the wild type level or decreased despite most of these variants increasing Arg-Asp formation. The most significant outlier was S84L in that it increased specificity to a ratio of 2.4 like S84F, but severely reduced main product formation as well.

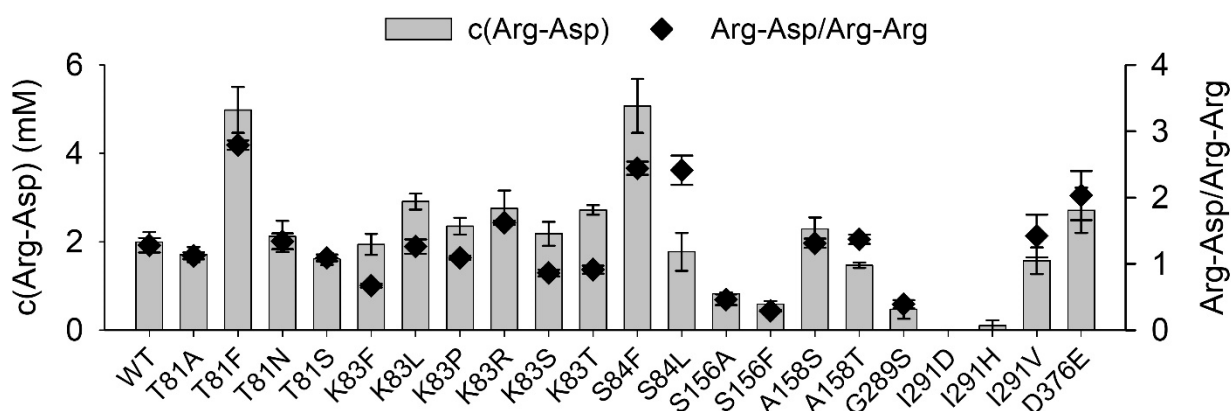


Figure 5. Formation of Arg-Asp and Arg-Arg by the RizA variants. 30 mM of both arginine and aspartic acid and 20 h reaction time at 25 °C. Reactions were set up in triplicates and data is presented as mean \pm standard deviation.

2.2.4. Arginine + Serine

Wild type RizA displayed the highest observed product formation with serine producing 10.0 mM Arg-Ser and a yield of 33% (Figure 6). Like for aspartic acid, T81F and S84F showed the highest increases in product formation to 14.1 mM and 12.5 mM, respectively. With the exception of S84L, S156A/F and A158T, all mutations in the C-terminal binding pocket retained most of the activity of the wild type enzyme or increased it. All mutations in the N-terminal binding pocket decreased product formation or prevented it, as was the case for I291D and I291H. Regarding side product formation, wild type RizA displayed the highest specificity for Arg-Ser versus Arg-Arg among the examined amino acids, producing Arg-Ser at a ratio of 7.8 to Arg-Arg (Figure 6). In the variants, these ratios varied between 3.2 in G289S and 11.6 in S84F, which were also the variants with the lowest/highest detected Arg-Ser concentrations, respectively (Figure 4). S84L also increased the ratio to 10.9, again in combination with reduced activity. Similarly, I291V lowered the activity but increased specificity to 9.3. Similar to the reactions with aspartic acid, K83F/S/T increased product formation but lowered specificity. While T81F showed the largest increase in main product formation, it only displayed a small increase to 8.3.

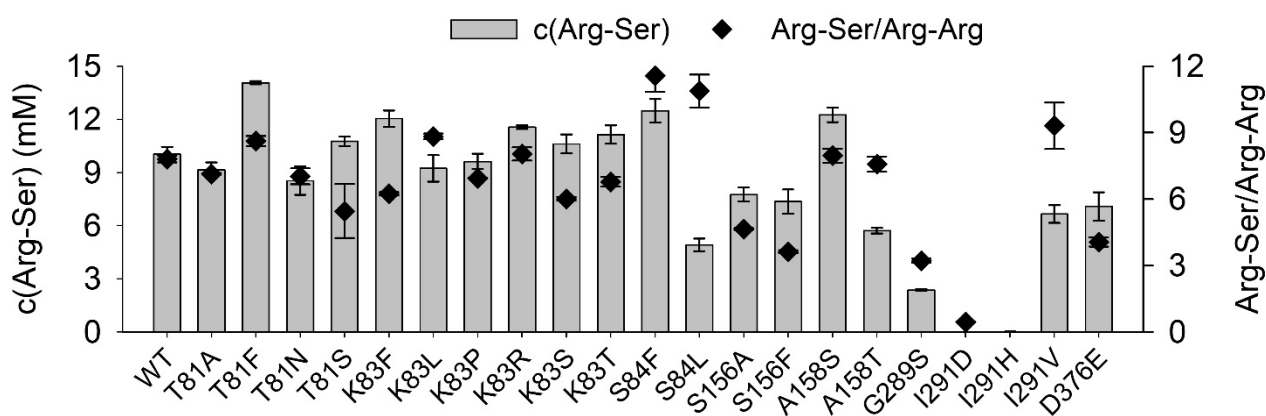


Figure 6. Formation of Arg-Ser and Arg-Arg by the RizA variants. 30 mM of both arginine and serine and 20 h reaction time at 25 °C. Reactions were set up in triplicates and data is presented as mean \pm standard deviation.

2.2.5. Arginine + Alanine

Arg-Ala was produced by the wild type at a concentration of 3.8 mM (13% yield) and the variants ranged from 1.2 mM in G289S to 5.6 mM in D376E (Figure 7). Apart from D376E, T81F and S156F also increased product concentration. Most mutations in the C-terminal binding pocket retained activity comparable to the wild type enzyme with the exception of S84L, which only produced 1.7 mM Arg-Ala. In contrast to D376E, all other mutations in the N-terminal binding pocket were detrimental to the formation of Arg-Ala. Most variants retained an Arg-Ala/Arg-Arg ratio comparable to the wild type (1.8) or below with K83F showing the lowest detected specificity of 1.2 (Figure 7). S84L and I291V had the highest specificity of 2.7, but at the expense of overall activity.

2.2.6. Arginine + Phenylalanine

Among the examined amino acids, phenylalanine showed the second lowest product concentration with 3.3 mM Arg-Phe and a yield of 11% using the wild type enzyme (Figure 8). In contrast, the variant S156A produced the highest heterodipeptide concentration in this study, with the exception of the serine reactions. In total, 12.1 mM Arg-Phe were produced corresponding to a yield of 40%. Other variants with significant increases were D376E, S156F, and K83F. Most other variants performed on the wild type level or below. S156A displayed the highest increase in specificity observed in this study from a ratio of 1.4 with wild type RizA to 9.7 in this variant (Figure 8). D376E and S156F showed

increased ratios, but K83F did not display a significant difference in ratio despite increased formation of Arg-X indicating a higher production of Arg-Arg as well.

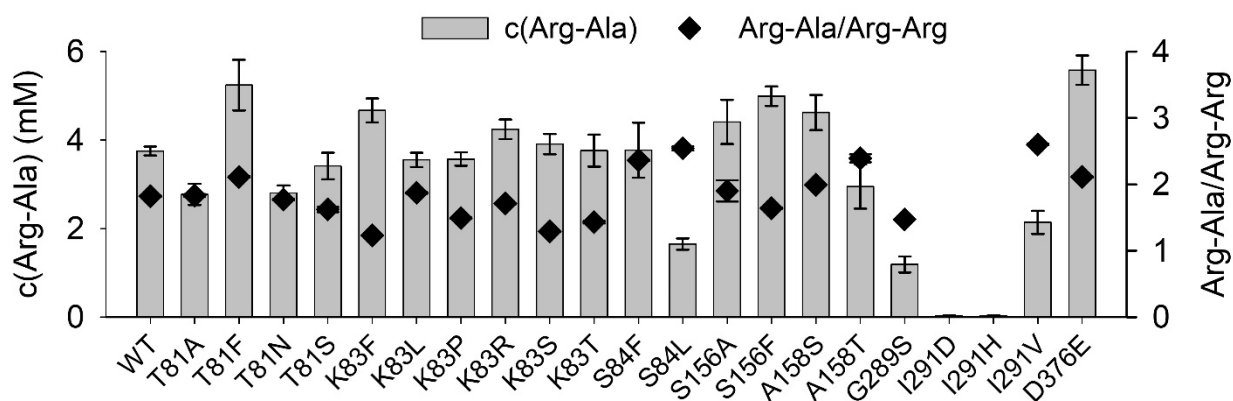


Figure 7. Formation of Arg-Ala and Arg-Arg by the RizA variants. 30 mM of both arginine and alanine and 20 h reaction time at 25 °C. Reactions were set up in triplicates and data is presented as mean \pm standard deviation.

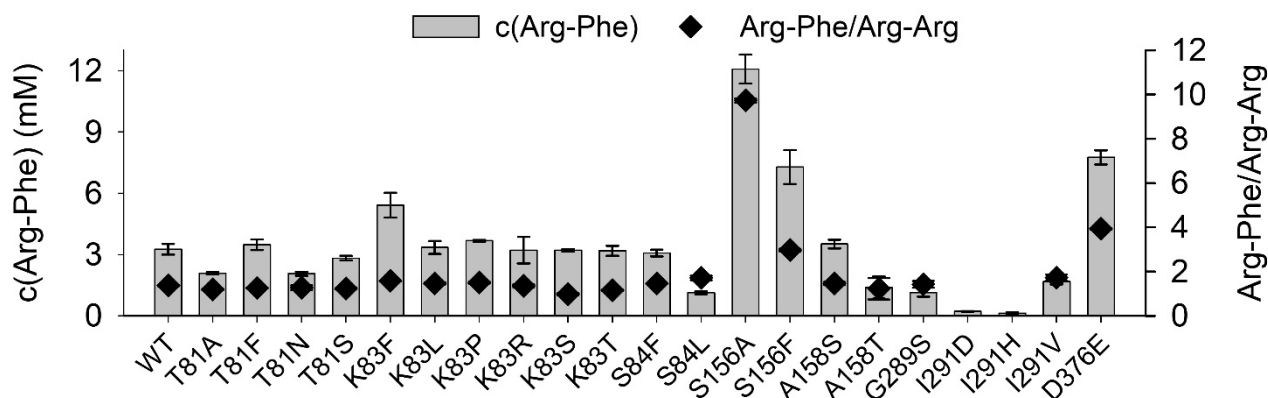


Figure 8. Formation of Arg-Phe and Arg-Arg by the RizA variants. 30 mM of both arginine and phenylalanine and 20 h reaction time at 25 °C. Reactions were set up in triplicates and data is presented as mean \pm standard deviation.

2.3. Effects of Mutations on the Substrate Specificity and Biocatalytic Productivity of RizA

To analyze the effect of the mutations on the substrate specificity and biocatalytic performance, the percentage change of Arg-X product formation was calculated for each substrate and variant in comparison to the wild type enzyme (Figure 9). Additionally, a 3D bar chart summarizing dipeptide formation for all amino acid combinations is available in Figure S2.

T81A/N/S had similar effects and generally increased Arg-Arg production, but decreased the other dipeptide concentrations by up to 37%. In contrast, T81F increased product formation for all amino acids except phenylalanine significantly up to 150% for aspartic acid. BI-LAL also contains a phenylalanine at the respective position (Figure 2) and was found to prefer small amino acids like serine or alanine as its C-terminal substrate [23]. TabS has been shown to accept serine as a major C-terminal substrate and—like wild type RizA—contains a threonine at this position in line with the similar high affinity of the wild type enzyme for serine [27].

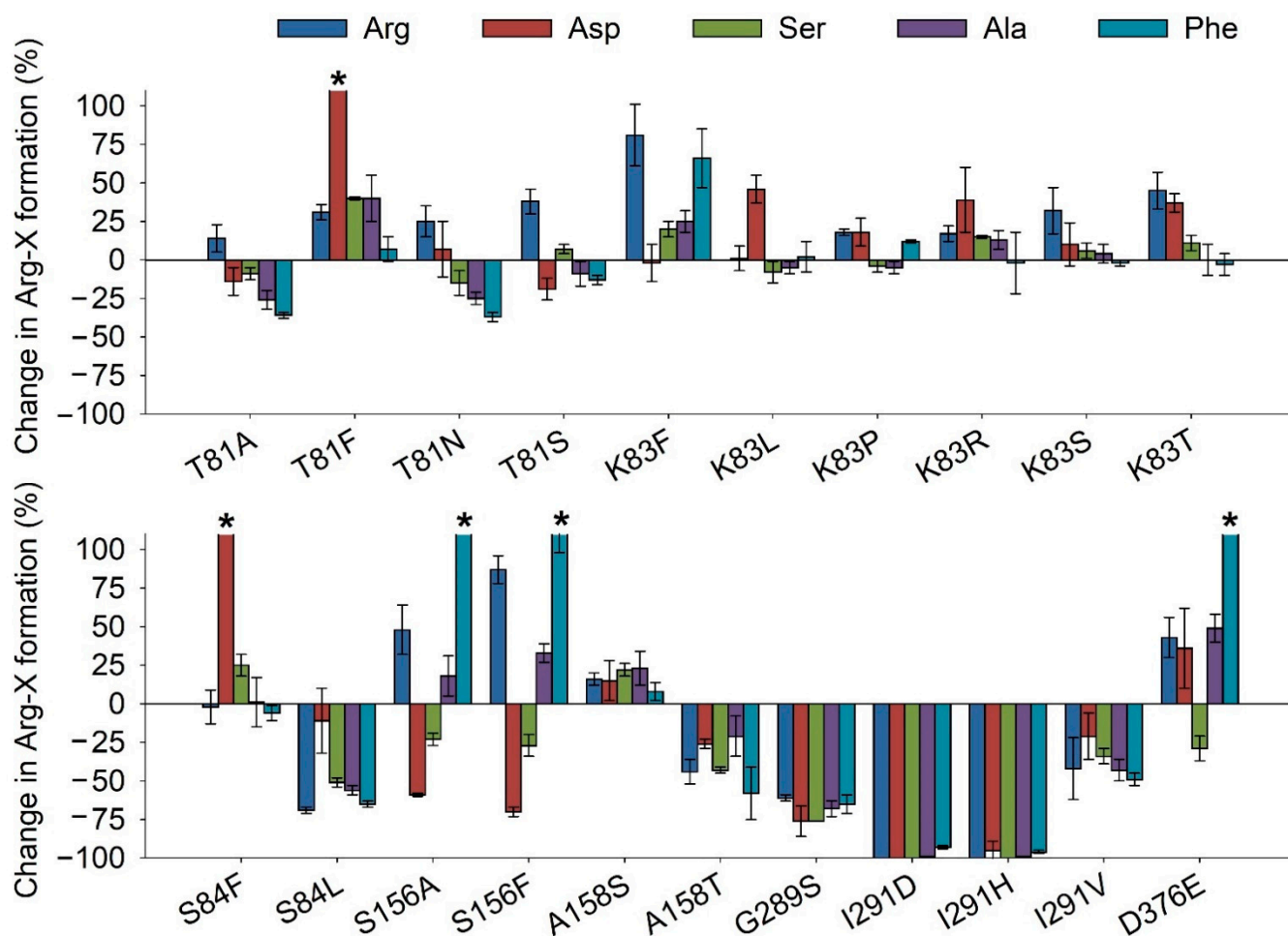


Figure 9. Change in product formation with RizA variants compared to the wild type with each substrate. * Values over 100% were truncated: T81F with Asp: $150 \pm 26\%$; S84F with Asp: $155 \pm 31\%$; S156A with Phe: $270 \pm 22\%$; S156F with Phe: $123 \pm 25\%$; D376E with Phe: $138 \pm 11\%$ (see Figure S3 for graph without truncation).

Similar to position 81, the substitution to phenylalanine at position 83 had a larger effect than the other variants K83L/P/R/S/T, where only small effects on product formation with serine, alanine and phenylalanine were detected, while for arginine and aspartic acid it increased by up to 46%. K83F had no effect on the aspartic acid reactions, but increased product formation for serine and alanine by about 20%, by 66% with phenylalanine and finally by 81% with arginine making it the variant with the highest Arg-Arg production alongside S156A. In line with this, K83F also showed increased Arg-Arg formation as a side product in the reactions with a second amino acid as seen by the lowered specificity ratios (Figures 5–7). A possible explanation for this large effect is that the mutation of lysine to phenylalanine removed a positive charge from the substrate binding pocket, which normally repels the positive charge of the guanidinium moiety of the arginine substrate. However, the other K83 variants also removed this positive charge, so the substitution to phenylalanine must have an additional positive effect on the binding of arginine apart from the charge.

At position 84, substitution to leucine had a detrimental effect on reactions with all amino acids except aspartic acid, where no significant effect was registered. In contrast, S84F dramatically increased Arg-Asp formation by 155% making it the best variant for this dipeptide alongside T81F. Arg-Ser production was also increased by 25% with no significant effect on the other amino acids. Both S84F and S84L showed large increases in the specificity ratios for most reactions (Figures 5–7). However, S84F mainly increased main product formation and side product formation remained relatively unchanged, while

for S84L main product formation decreased, but side product formation decreased even further resulting in an overall increased specificity. This is also reflected in the reactions containing only arginine, where S84F had no detected effect on Arg-Arg formation, but S84L decreased it by 69%.

Substitution of the serine at position 156 to alanine or phenylalanine had similar effects on the substrate specificity, as in both cases product formation with aspartic acid and serine was lowered, and product formation with arginine, alanine, and phenylalanine increased, but the variants varied in how severe the decreases/increases were. For example, S156F increased Arg-Phe production by 123%, whereas S156A increased it by 270%. The large increase for phenylalanine correlated with BacD and TabS, which also contain an alanine at the homologous position and accept phenylalanine readily as the C-terminal substrate [20,27]. However, RsLAL also contains alanine but prefers “non-bulky” C-terminal substrates [22]. In general, it is plausible that a smaller, non-polar residue like alanine would be more suitable than serine for a large, non-polar substrate like phenylalanine. The mutation to phenylalanine also reduced the polarity of the substrate binding pocket, but its larger size and thus reduced space in the binding pocket could be an explanation for the smaller increase with the large substrate.

Position A158 was substituted by serine or threonine. Despite the small difference between these two amino acids, they had opposite effects: A158S increased product formation for all amino acids by 8 to 23% and A158T decreased it by 21 to 58%. It was noted that position 158 cannot be unambiguously assigned to the C-terminal binding pocket and that it seems to be oriented more towards the N-terminal binding pocket in the BacD structure (Figure 2a). Due to its position, it might be affecting the overall substrate binding more than the N- and C-terminal substrate specifically.

In the N-terminal binding pocket, the mutations of G289 and I289 all drastically reduced product formation for all examined amino acids. Significant differences between C-terminal amino acids were not observed, as expected from their position in the N-terminal binding pocket. Likely, these mutations inhibited binding of the N-terminal arginine, which is the first step of the catalytic cycle and thus inhibited product formation with all examined amino acids [30]. D376E was the only mutation in the N-terminal binding pocket with increases in product formation. While Arg-Ser decreased, Arg-Arg, Arg-Asp, and Arg-Ala increased by 36 to 49% and Arg-Phe by 138%, respectively, thus making it the second-best variant for Arg-Phe after S156A.

Data of the biocatalytic performance for the two best variants in terms of yield and specificity for each amino acid is summarized in Table 1.

Table 1. Variants with the highest biocatalytic performance for the production of Arg-X dipeptides.

| Amino Acid X | Variant | c (Arg-X) (mM) | | c (Arg-Arg) (mM) | | Arg-X/Arg-Arg | Yield (Arg-X) (%) |
|---------------|-----------|----------------|-------|------------------|-------|---------------|-------------------|
| Arginine | Wild type | 5.6 | ± 0.7 | - | - | - | 19 |
| | S156F | 10.5 | ± 0.5 | - | - | - | 35 |
| | K83F | 10.2 | ± 1.1 | - | - | - | 34 |
| Aspartic acid | Wild type | 2.0 | ± 0.2 | 1.6 | ± 0.2 | 1.3 | 7 |
| | T81F | 5.0 | ± 0.5 | 1.8 | ± 0.2 | 2.8 | 17 |
| | S84F | 5.1 | ± 0.6 | 2.1 | ± 0.2 | 2.4 | 17 |
| Serine | Wild type | 10.0 | ± 0.4 | 1.3 | ± 0.1 | 7.8 | 33 |
| | T81F | 14.1 | ± 0.1 | 1.6 | ± 0.0 | 8.6 | 47 |
| | S84F | 12.5 | ± 0.7 | 1.1 | ± 0.1 | 11.6 | 42 |
| Alanine | Wild type | 3.7 | ± 0.1 | 2.1 | ± 0.1 | 1.8 | 12 |
| | D376E | 5.6 | ± 0.3 | 2.6 | ± 0.2 | 2.1 | 19 |
| | T81F | 5.2 | ± 0.6 | 2.5 | ± 0.3 | 2.1 | 17 |
| Phenylalanine | Wild type | 3.3 | ± 0.3 | 2.4 | ± 0.2 | 1.4 | 11 |
| | S156A | 12.1 | ± 0.7 | 1.2 | ± 0.1 | 9.7 | 40 |
| | D376E | 7.8 | ± 0.4 | 2.0 | ± 0.1 | 3.9 | 26 |

T81F was among the best variants for all three examined small amino acids. In the case of the small, polar amino acids aspartic acid and serine, S84F was the second-best variant. Considering the close proximity and orientations of T81 and S84 to each other (Figure 3), it is plausible that the same substitution led to similar effects in T81F and S84F. Overall, decreasing the size of the C-terminal binding pocket with mutations to phenylalanine (T81F, K83F, S84F and S156F) generally increased product formation for small amino acids, although not uniformly. For example, the serine at position 156 possibly had a beneficial effect for the binding of small, polar amino acids, as both substitutions (S156A/F) decreased product formation for serine and aspartic acid. The residue might have a discriminating effect on the polarity of the substrate, as both substitutions instead increased product formation with the non-polar alanine and phenylalanine. Both of these substrates also shared D376E as one of the best variants (Table 1). It was the only mutation in the N-terminal binding pocket that had positive effects on product formation. If the hypothesized function of D376 in binding the N-terminal arginine by electrostatic interaction [28] is correct, then it stands to reason that D376E is also capable of that function, but that it might change the orientation of the substrate as a whole due to the larger size of glutamic compared to aspartic acid. This could explain why it was the only mutation in the N-terminal binding pocket significantly affecting C-terminal specificity.

For all substrates, variants with significantly improved biocatalytic performance were identified (Table 1). Alanine was the substrate with the smallest increases from 12 to 19% yield in D376E and only a modest increase in the specificity ratio Arg-X/Arg-Arg. Aspartic acid was the substrate with the lowest yield, and Arg-X/Arg-Arg ratio in the wild type and both parameters were increased more than two-fold in T81F. Serine was the best among the wild type RizA substrates and was still substantially improved from a yield of 33 to 47% and a small increase in specificity in T81F. In addition, S84F significantly increased the specificity to 11.6. Phenylalanine was the substrate with the second-lowest yield of 11% and specificity of 1.4 after alanine. Conversely, S156A conferred the largest increases for both parameters observed in this work and increased them to 40% and 9.7, resulting in a similar performance than observed with serine.

While the yields for Arg-Ala and Arg-Asp were still relatively low despite the large relative change in yield, T81F and S156A represent suitable candidates for the production of the salt-taste enhancer Arg-Ser and the potential antihypertensive Arg-Phe using RizA. In comparison, the previously highest Arg-Ser yield was 41% using higher wild type RizA concentrations and 50 mM amino acids concentration [26]. Arg-Phe has been previously produced with TabS with a yield of 62% using 12.5 mM amino acids. S156A represents a single mutation drastically changing the substrate specificity. However, redesign of the substrate specificity of LALs is likely a more complicated matter not to be solved by single mutations.

Apart from completely random directed evolution approaches, protein engineering always benefits from knowledge on the relationship of protein sequence and function in the form of reaction data, structures, and mutagenesis studies [19,33]. In the present work, a total of eight positions were examined and targeted for their role in substrate specificity. It represents the first mutagenesis study of RizA and confirmed the importance of positions 81 and 83 for substrate specificity that were already explored in other LALs. Furthermore, the positions S84, S156, and A158 have never been examined in any LAL regarding their potential for changing the substrate specificity. RizA is both an excellent study object of LAL substrate specificity and the production of arginyl dipeptides due to its high N-terminal specificity and relaxed C-terminal specificity. With the gained knowledge, RizA can be further engineered by combining mutations or performing saturation mutagenesis at the identified positions, as has been previously done for the production of Pro-Gly with TabS [24]. While the specificity of Arg-X versus Arg-Arg formation has been substantially improved in the created variants (Table 1), a further reduction of this side product would be a suitable engineering target. Finally, since proline is the only amino acid not accepted by RizA, and Arg-Pro is the strongest salt-taste enhancer among

the arginyl dipeptides [11], adapting RizA for the production of this dipeptide is another worthwhile challenge. A promising approach is the use of protein modelling to guide the selection of mutations, which has been successfully performed recently for the engineering of BaLAL [31]. New tools like the AI-based AlphaFold software [34] allow for the prediction of the 3D-structure of a protein from its primary sequence. However, enzymes undergo conformational changes while binding the substrate, a process based on a mutual stereochemical fit and a variety of molecular interactions. Until all of these conformational states and interactions can be precisely modeled, experiments as described above will be indispensable for understanding the relationship of protein structure and function.

3. Materials and Methods

3.1. Chemicals, Reagents and Strains

All chemicals were purchased from Carl Roth (Karlsruhe, Germany) or Sigma Aldrich (Taufkirchen, Germany) if not otherwise indicated. Enzymes for molecular biology were purchased from Thermo Fisher Scientific (St. Leon-Roth, Germany). The pET28a vector was purchased from Merck KGaA (Darmstadt, Germany). The *E. coli* strains BL21 (DE3) and TOP10 were maintained in our laboratory. Oligonucleotides were synthesized by Microsynth Seqlab GmbH (Goettingen, Germany).

3.2. Mutagenesis of RizA

Structural alignment and images of protein structures were created with PyMOL version 2.4.0 (2020) by Schrödinger, LLC (New York, NY, USA) [29]. Sequence alignments were created with SnapGene version 5.1.7 (2020) from GSL Biotech LLC (Chicago, IL, USA). Mutagenesis of the pET28_his6-rizA construct [26] was performed by whole-plasmid PCR using overlapping, mutagenic primers, and was adapted from Liu and Naismith [35]. PCR were performed in a Biometra thermal cycler from Analytik Jena (Jena, Germany) using Phusion DNA polymerase from Thermo Fisher Scientific (St. Leon-Roth, Germany) and the pET28a_his6-rizA plasmid as template (50 ng). Primers (Table S1) were designed with the software SnapGene version 5.1.7 (2020) from GSL Biotech LLC (Chicago, IL, USA). The three-step protocol started with a denaturing step at 98 °C for 30 s, then 20 cycles of 98 °C for 10 s, the annealing temperature (Table S1) for 30 s and 72 °C for 130 s. Lastly, final extension was performed at 72 °C for 10 min and PCR products were stored at 8 °C. In the two-step protocol (Table S1), the elongation step at the annealing temperature was omitted. PCR products were visualized by agarose gel electrophoresis and digested by addition of 1 µL DpnI to 50 µL PCR and incubation at 37 °C for 2 h followed by inactivation at 80 °C for 5 min. The digested reactions were transformed into chemocompetent *E. coli* TOP10. After confirming the correct mutation through sequencing by Microsynth Seqlab GmbH (Goettingen, Germany), the variant plasmids were transformed into *E. coli* BL21 (DE3) for expression and glycerol stocks were stored at −80 °C.

3.3. Cultivation and Purification

Cultivation and purification were performed as previously described for wild type RizA [26]. In short, for each variant 100 mL TB medium were inoculated and incubated at 37 °C/160 rpm until induction and harvested after 20 h expression at 20 °C/160 rpm. After cell disruption by sonication, the RizA variants were purified by affinity chromatography and desalted by gel filtration.

3.4. Biocatalysis

Biocatalytic reactions were performed according to the previously described conditions [26]. The reactions were performed in 0.2 mL PCR tubes with 50 µL reaction volume and contained 30 mM arginine and the second amino acid, 30 mM AcP, 0.5 mM ATP, 7.5 mM MgSO₄, 0.2 mg/mL RizA, 0.1 mg/mL AckA and 25 mM tricine buffer pH 8.5. Reactions and substrate controls containing no RizA were set up in triplicates and incubated at 25 °C

in a Biometra thermal cycler from Analytik Jena (Jena, Germany). After 20 h, the reactions were inactivated at 70 °C for five min and stored at −20 °C until analysis.

3.5. Analysis

The amino groups of dipeptides were derivatized with *o*-phthalaldehyde and analyzed by RP-HPLC with fluorescence detection with the same analytical setup described by Rottmann, et al. [36]. Similar to the previous study on RizA [26], the gradient consisting of eluent A (0.1 M sodium acetate buffer pH 6.5) and eluent B (Methanol HPLC grade) was modified for the measurement of dipeptides. Two different gradient programs were used. For the measurement of Arg-Asp, Arg-Ser, and Arg-Ala alongside Arg-Arg (gradient 1), the gradient was as follows: 0 min: 70% A, 5 min: 70% A, 10 min: 60% A, 15 min: 0% A, 20 min: 0% A, 25 min: 70% A, 30 min: 70% A. For the measurement of Arg-Phe alongside Arg-Arg (gradient 2), the gradient was as follows: 0 min: 60% A, 5 min: 60% A, 10 min: 50% A, 15 min: 0% A, 20 min: 0% A, 25 min: 60% A, 30 min: 60% A. Exemplary chromatograms for each gradient program are shown in Figures S3 and S4. Beta-alanine was added as an internal standard for the derivatization. External calibration was performed with analytical standards of the dipeptides Arg-Arg, Arg-Asp, Arg-Ser, Arg-Ala, and Arg-Phe, which were purchased from Bachem (Bubendorf BL, Switzerland). All standard solutions were set up in triplicate. Data was visualized with SigmaPlot 14.5 (2020) from Systat Software GmbH (Erkrath, Germany).

4. Conclusions

Mutagenesis of RizA improved the yield and substrate specificity for the production of bioactive dipeptides. The gained data both confirmed the roles of several residues already implicated in substrate specificity and identified new ones that can be used in future protein engineering studies.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/catal11111385/s1>, Figure S1: 3D bar chart of dipeptide formation, Figure S2: Change in product formation of RizA, Figure S3: Exemplary chromatogram with gradient program 1 for measurement of Arg-Ser, Figure S4: Exemplary chromatogram with gradient program 2 for measurement of Arg-Phe, Table S1: Primer pairs for mutagenesis.

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