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Genetically Fused T4L Acts as a Shield in Covalent Enzyme Immobilisation Enhancing the Rescued Activity

Matteo Planchestainer, David Roura Padrosa, Martina Letizia Contente and Francesca Paradisi *

School of Chemistry, University of Nottingham, University Park, Nottingham NG7 2RD, UK; matteo.planchestainer@nottingham.ac.uk (M.P.); pcxdr1@exmail.nottingham.ac.uk (D.R.P.); martina.contente@nottingham.ac.uk (M.L.C.)

* Correspondence: francesca.paradisi@nottingham.ac.uk; Tel.: +44-(0)115-74-86267

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Abstract: Enzyme immobilisation is a common strategy to increase enzymes resistance and reusability in a variety of excellent 'green' applications. However, the interaction with the solid support often leads to diminished specific activity, especially when non-specific covalent binding to the carrier takes place which affects the delicate architecture of the enzyme. Here we developed a broadly applicable strategy where the T4-lysozyme (T4L) is genetically fused at the N-terminus of different enzymes and used as inert protein spacer which directly attaches to the carrier preventing shape distortion of the catalyst. *Halomonas elongata* aminotransferase (HEWT), *Bacillus subtilis* engineered esterase (BS2m), and horse liver alcohol dehydrogenase (HLADH) were used as model enzymes to elucidate the benefits of the spacer. While HEWT and HLADH activity and expression were diminished by the fused T4L, both enzymes retained almost quantitative activity after immobilisation. In the case of BS2m, the protective effect of the T4L effectively was important and led to up to 10-fold improvement in the rescued activity.

Keywords: aminotransferase; esterase; alcohol dehydrogenases; biocatalysis; enzyme immobilisation

1. Introduction

With the current expansion of biocatalysis and the consequent extensive application of enzymes to chemistry manufacturing, solutions have been introduced to overcome the issues caused by the low stability of these natural catalysts [1,2]. In this panorama, immobilisation offers the significant advantage of rendering enzymes robust and reusable 'green' tools, which allow for the development of efficient biotransformations [3,4]. Enzyme immobilisation is indeed a very common solution to improve enzymatic stability and to make it less sensible to harsh environments (temperature, solvents, different pH) typical of synthetic reaction conditions [5].

Immobilisation techniques involve the interaction between a solid support, the carrier, and the enzyme. An essential requirement for any carrier is a large surface area which can be obtained using small porous beads. The physical characteristics of the matrices, the material (natural or synthetic, organic or inorganic), and the surface properties play an important role in the efficient binding of the enzymes [6] and the catalytic features of the immobilised catalyst. Although the immobilised enzymes show generally a satisfactory improvement in their stability, the loss of performance is significant compared to the free protein [7,8]. This effect is almost universally observed, especially among covalently immobilised enzymes, and it is mainly attributed to a severe protein shape distortion that develops during the process [9]. Several strategies have been described in the literature to overcome

these limitations, however, they often shy away from covalent linking and are directed to non-covalent immobilisation strategies and interactions [6,10].

Despite the loss of catalytic efficiency, covalent immobilisation is still often a method of choice as it prevents leaching of the enzyme in the reaction. Molecular spacers, such as glutaraldehyde, have been satisfactorily applied in minimising enzyme deactivation [11]. Interestingly, in literature there are no examples where a second protein is used as a spacer for covalent immobilisation.

Chimeric structures where a second protein is fused to a target macromolecule (i.e., enzymes, receptors, etc.) have been successfully reported for a variety of applications such as increasing protein solubility, purification strategies, labelling, and crystallization [12]. An excellent example was the use of the lysozyme from the bacteriophage T4 (T4L), a small (160 amino acids), globular, highly soluble, protein which was exploited as fused chimeric system in several remarkable experiments of X-ray crystallography as a crystallization promoting agent, and it allowed for the first time to elucidate the structure of G-protein coupled receptors (GPCRs) [13]. In the context of enzyme immobilisation, a protein linker has been described for a non-covalent attachment driven by the insertion of a cationic binding module ($Z_{basic}2$) as an additional domain. However, in this case the fused protein was mainly exploited to promote the interaction towards an anionic carrier (CPG-sulfonate) [14,15].

In this work, we investigated the use of a protein linker in covalent enzyme immobilisation with the scope of maximising the rescued activity. The one-pot strategy developed for covalent immobilisation by Guisan et al. [16] exploits a polyhistidine tag, commonly used for ion metal affinity chromatography (IMAC) purification, to selectively direct the enzyme on metal-activated supports thus allowing for a faster reaction between the reactive matrix moieties (i.e., epoxy groups) and the nucleophilic lysine of the protein. This technique is reliable and broadly applied, however the random distribution of lysines on the enzymatic surface prevents rational attachment, and the rescued activity, while commonly expected to be lower than the free enzyme, is unpredictable. The genetic insertion of T4L, naturally rich in lysines, between the His-tag and the target enzyme would more favourably form covalent bonding, possibly preventing any direct cross-linking between the target enzyme and the carrier, leaving the enzyme solvent exposed.

The strategy was initially tested on the well characterised *Halomonas elongata* aminotransferase (HEWT) [17] which we have used covalently immobilised for biotransfomations in continuo; however, upon direct cross-linking to the carrier, the recovered activity reached about 30% when compared to the free enzyme [18,19]. T4L mediated immobilisation was then extended to horse liver alcohol dehydrogenase (HLADH) [20] and to a *Bacillus subtilis* esterase (BS2m) engineered (I270F/F314Y) to enhance its amidase activity [21].

2. Results

2.1. Construction of the Chimeras

A plasmid harbouring the T4L gene was exploited as vector backbone for the sub-cloning of the three target enzymes. The T4L was engineered by point mutation to render it catalytically inactive (E11Q mutation) in order to prevent any possible interference with the enzymatic activity of the selected catalysts. Kobilka and co-workers demonstrated that a relatively flexible linker may be necessary to allow the receptor and the T4L to fold correctly [22]. Therefore, a first construct was designed to incorporate a linker of nine amino acids between the T4L and the protein exploiting the existing amino acid sequence already present in the pRSET-b plasmid between the His-tag and the multi-cloning site (MCS). Furthermore, to evaluate the effect of the rigidity induced by the peptide loop between the two proteins, a second construct was created, reducing the existing spacer from nine to three amino acids (Figure 1).

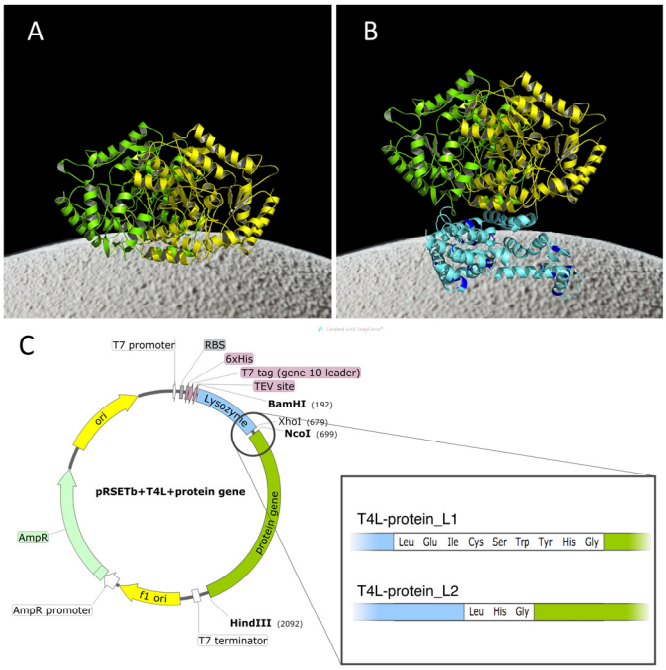

Figure 1. Proposed architecture of HEWT (green and yellow represent the two enzyme subunits) in covalent bonding directly on the solid support surface (**A**) or mediated by a linker protein (light blue, in dark blue the reactive lysines) (**B**). pRSET-b construct map outlining T4L (blue) and the protein gene (green). The inset shows the sequences of the two aminoacidic linkers separating the lysozyme and the target protein (**C**).

2.2. Halomonas elongata Aminotransferase

HEWT gene (*spuC*) was trialled first in both constructs (T4L-HEWT_L1 and T4L-HEWT_L2, nine and three amino acid long spacers, respectively). The expression level of both chimeras was lower than what obtained for HEWT, resulting in 12 and 20 mg of protein per litre of media, after IMAC purification, for T4L-HEWT_L1 and L2, respectively (Table 1). The proteins analysed by SDS-page electrophoresis did not show formation of any inclusion bodies caused by incorrect folding, and the decrease expression might simply be attributed to physiological limitation of the *E. coli* host in translating a longer gene. Although fusion tags have been reported to have a positive influence on protein solubility and expression, they do not function equally well with all target proteins [12]. Furthermore, analysis of the purified fractions confirmed the integrity of the constructs showing a molecular weight in the correct range of expected mass (Figure S1). HEWT specific activity was also affected by the presence of the lysozyme, with values of 1.37 and 0.50 U/mg for the two T4L-HEWT (Table 1). Such an effect could be explained with the fact that the N-term is a delicate structural domain which forms part of the active site; an exogenous module linked to it has a direct influence on its position and consequently on its catalytic efficiency. Indeed, with the longer linker the effect on activity was less severe, while with only three amino acids separating the lysozyme from HEWT the activity dropped 10 times with respect to the control. The proximity of the T4L can also hinder the substrate accessibility to the active site further influencing the reactivity.

The immobilisation of the chimeric constructs was carried out on two supports: Sepabeads EC-EP/S are described to have an average pore diameter of 10–20 nm, and ReliZyme EP403/S with a larger pore size (20–40 nm) which should favour better protein distribution and prevent 'caging' of the enzymes into narrow pores. In fact, the additional bulk of the T4L makes the chimeric construct significantly larger and this could potentially lead to immobilisation difficulties when the pore size of the resin is small. Immobilisation of the constructs followed the same procedure described

previously [18], maintaining cobalt(II) as the coordinating metal. Initially, 5 mg of enzyme per gram of resin were used, allowing the protein to react with the epoxy groups on the carrier for 24 h (Table 1).

Table 1. Comparison of specific activities and expression levels of the original *Halomonas elongata* aminotransferases (HEWT) and the two chimeric proteins in the free enzyme form. Specific activities of the resin following immobilisation of enzymes (5 mg_{enzyme}/g_{resin}), and percentage of residual activity after immobilisation (24 h, room temperature, in 50 mM phosphate buffer pH 8) with respect to the free protein.

	Free Enzyme		Immobilised Enzyme onto Sepabeads EC-EP/S (Pore ø 10–20 nm)		Immobilised Enzyme onto ReliZyme EP403/S (Pore ø 20–40 nm)	
	Specific Activity (U/mg)	Expression (mg/L)	Specific Activity (U/g)	Rescued Activity (%)	Specific Activity (U/g)	Rescued Activity (%)
HEWT	4.51 ± 0.05	40	5.1 ± 0.8	22.3	5.8 ± 0.6	26.1
T4L-HEWT_L1	1.37 ± 0.08	12	5.5 ± 0.5	80.4	6.0 ± 0.4	89.1
T4L-HEWT_L2	0.50 ± 0.07	20	1.5 ± 0.3	59.8	2.1 ± 0.1	84.1

On Sepabeads EC-EP/S, the control HEWT sample yielded 22% of recovered activity, while both T4L-HEWTs showed a marked improvement in the retention of activity with 80.4% and 59.8%, for the nine- and three-amino acid linker, respectively. These results confirm that the TL4 prevents direct attachment of the enzyme to the resin and this effect is enhanced by a longer spacer. On ReliZyme EP403/S the smaller wild type HEWT behaved virtually in the same manner, and likewise T4L-HEWT_L1 for which the rescued activity was already very high and the benefits of larger pores were not particularly significant. However, for the T4L-HEWT_L2 construct, the change observed in recovered activity was important, with almost a 30% improvement. Larger pores in this case may impact the number or covalent bonding with the surrounding surface leading to a reduced rigidity and distortion of the protein as well as generally increasing the substrate diffusion rate.

A range of concentrations for HEWT and T4L-HEWTs were then screened to assess the loading capacity of the resin, and whether the specific activity as well as the rescued activity could be further improved. The behaviour of HEWT and T4L + HEWT_L2 did not change significantly between 1 and 5 mg. At 10 mg of enzyme, the rescued activity decreased presumably due to surface overloading. On the other hand, the T4L + HEWT_L1 presents a bell-shape behaviour with a peak at 3 mg_{enzyme}/g_{resin} (Figure 2); it is plausible that the T4L coats the surface of the resin further protecting the enzyme from any binding to the resin yielding to almost equal activity as recorded for the free enzyme and a specific activity of the imm-T4L-HEWT_L1 of 1.28 U/mg versus 1.38 U/mg of the free T4L-HEWT_L1.

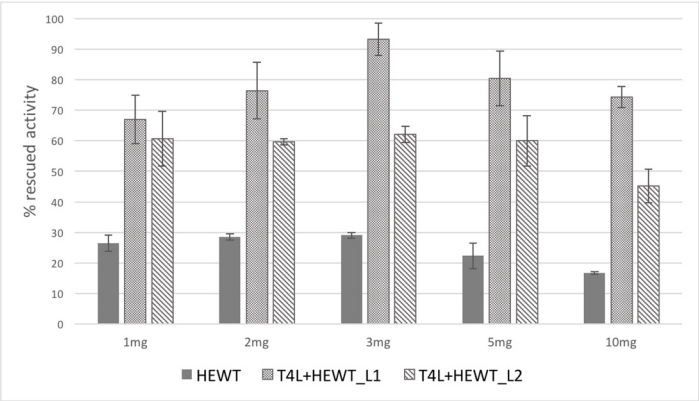

Figure 2. HEWT, T4L + HEWT_L1 and T4L + HEWT_L2 immobilisation (24 h, room temperature, in 50 mM phosphate buffer pH 8) at different enzyme loading per gram of resin Sepabeads EC-EP/S (pore ø 10–20 nm).

The incubation time to facilitate enzyme binding was investigated. It was postulated that the high distribution of lysines on the T4L surface could promote a faster immobilisation, and shorter incubation time may result in higher rescued activity. The immobilisation was thus carried out by leaving the enzymes to react with the resin between 1 and 24 h. Wild-type HEWT rescued activity was unaffected by the incubation time and consistent around 25–30%, while T4L + HEWT_L1 showed an unexpected behaviour with optimal value of rescued activity only after 24 h (Figure S2). 24 h was therefore selected as the ideal incubation time for further assays for all the HEWT variants.

Since the T4L chimeric proteins showed greater retained activities, they were further analysed to investigate the effect of the linker protein on the stability of the enzyme. Under standard storage conditions (4 °C, buffer phosphate pH 8, 0.1 mM PLP), the immobilised T4L-HEWTs showed unchanged specific activity for over a month. When the stability was evaluated by incubating the resin at 37 °C and 45 °C, no significant differences were observed with respect of the control HEWT immobilisation. On the other hand, the stability under working conditions (consecutive cycles of bioconversion), showed a slight negative trend in performance for the T4L-HEWT_L1 (Figure S3—circle), while the T4L-HEWT_L2 (Figure S3—square) was more stable with a virtually unchanged activity after 10 cycles, similarly to the imm-HEWT behaviour [18].

A significant feature of the imm-HEWT was the improved ability to withstand the presence of organic co-solvent in the reaction media with respect to the free enzyme. The construct harbouring the lysozyme spaced by the longer linker showed a similar behaviour to the free enzyme (Figure S4). This is not unexpected since the rationale of the protein linker was to prevent a direct bonding of the enzyme to the resin allowing a greater degree of freedom to the catalyst, which in this way no longer benefits from the imposed rigidity of the multi-point attachment to the carrier. However, when the shorter linker construct was assessed, the catalyst performed marginally better than the imm-HEWT (Figure S4), which is indicative of an enhanced stability induced by the close proximity of T4L to protein, similarly to the effect that BSA may have on pure proteins [23].

2.3. *Bacillus subtilis* Esterase

To validate the approach of the protein linker as a beneficial addition in enzymatic immobilisation, the strategy was extended to a second enzyme: *Bacillus subtilis* esterase, specifically a mutant variant (BS2m) engineered to augment its amidase activity [21]. Immobilisation of the purified enzyme via covalent attachment proved to be virtually impossible with almost complete loss of activity yielding negligible nominal activity of the support (1.3 U/g) when 5 mg_{enzyme}/g_{resin} were applied, equal to a rescued activity of 0.4% (Table 2). Such inability to retain activity upon covalent immobilisation is characteristic of many esterases [24,25]. Gross and co-workers speculated that this class of proteins tend to create a superficial layer preventing an efficient substrate diffusion [26]. As an example, the commercial catalyst NovoZyme 435 (*Candida antarctica* lipase-B, CAL-B) is immobilised at 82 mg_{enzyme}/g_{resin} but yields only 7.5% recovery activity, highlighting how this class of enzyme is particularly affected by immobilisation [27]. In addition, in the case of the BS2m variant, the superficial lysines, are concentrated in proximity to the active site which could drive the orientation of the enzyme in such a way that the catalytic pocket faces the support limiting its accessibility.

The same strategy applied for the HEWT was thus evolved towards the BS2m creating two chimeric constructs harbouring the 9- and 3-amino acid linkers. In this particular case, both longer and shorter linkers (T4L-BS2m_L1 and T4L-BS2m_L2, respectively) showed a less severe reduction in expression levels when compared to the original BS2m (Table 2, Figure S5) and an activity of 31 and 35 U/mg, respectively (Table 2). Covalent immobilisation onto the Sepabeads EC-EP/S epoxy-resin of the T4L-BS2m_L1 yielded an active resin with 5.5 U/g equal to 3.6% recovery activity, a notable 10-fold improvement on the original BS2m (Table 2). T4L-BS2m_L2, as observed for HEWT, did not perform as well as the L1 linker, with lower, yet still improved, immobilisation qualities (2.9 U/g and a 1.7% recovery activity). The co-immobilisation of T4L and BS2m, separately expressed and purified, was also tested to exclude a possible non-specific effect of the T4L but no enhancement of the

recovered activity was noted in this case (data not shown). This further confirmed that the fused T4L physically shields the enzyme from covalent attachment and does not directly affect enzyme activity.

Table 2. Comparison of specific activities and expression levels of the original *Bacillus subtilis* esterase (BS2m) and the two chimeric proteins in the free enzyme form. Specific activities of the resin following immobilisation of enzymes (5 mg_{enzyme}/g_{resin}), and percentage of residual activity after immobilisation (2 h, room temperature, in 50 mM phosphate buffer pH 8) with respect to the free protein.

	Free Enzyme		Immobilised Enzyme onto Sepabeads EC-EP/S (Pore ø 10–20 nm)		Immobilised Enzyme onto ReliZyme EP403/S (Pore ø 20–40 nm)	
	Specific Activity (U/mg)	Expression (mg/L)	Specific Activity (U/g)	Rescued Activity (%)	Specific Activity (U/g)	Rescued Activity (%)
BS2m	61 ± 4	30	1.3 ± 0.1	0.4	1.6 ± 0.3	0.5
T4L-BS2m_L1	31 ± 3	15	5.5 ± 0.8	3.6	6.2 ± 0.8	3.9
T4L-BS2m_L2	35 ± 2	13	2.9 ± 0.6	1.7	3.7 ± 0.4	2.1

Immobilisation of BS2m, T4L-BS2m_L1, and L2 was also tested on the ReliZyme (EP403/S). Interestingly, the new support offered better performance than the Sepabeads, allowing for a more efficient immobilisation for all variants (though still lower for the BS2m control). The recovered specific activity of T4L-BS2m_L1 reached 6.2 U/g (3.9%) and 3.7 U/g (2.1%) for the construct with the shorter link, confirming the benefits of a facilitated diffusion inside the solid support. This strategy therefore allowed to prepare an immobilised esterase with an activity considerably higher than the commercial NovoZyme 435 (4.5 U/g, tested under the same condition) using 16-fold less enzyme. Furthermore, the immobilised enzyme exhibited a great stability retaining 87% of its initial activity after five reaction cycles, comparable to the commercial CAL-B (83%) [27].

2.4. Horse Liver Alcohol Dehydrogenase

Finally, horse liver alcohol dehydrogenase (HLADH) was selected as an additional test enzyme, since its immobilisation had already been carried out on the same epoxy resin, providing a good reference for the experiment [28]. This enzyme presents additional challenges to general immobilisation strategies since its cofactor is not tightly bound and the cofactor binding domain needs to be accessible during the catalytic cycle [29]. For these reasons, structural alterations may have a major impact on the reactivity, unlike in the case of the aminotransferase where the PLP cofactor is permanently buried inside the active site.

The *adh* gene was subcloned into both vectors similarly to the other two genes. However, for both constructs, expression level and specific activity dropped dramatically with an activity 10 times lower, and yielding less than 1 mg of protein per litre of culture media after IMAC purification (Figure S6). Nevertheless, immobilisation of both chimeras and wild type HLADH was performed. The specific activity of the resin following immobilisation of the T4L-HLADHs (1 mg_{enzyme}/g_{resin}) was very low overall compared to the native imm-HLADH (Table 3). However, similarly to what observed for the HEWT, the T4L-shielded enzymes showed an improvement in the recovered activity although the overall nominal activity remained rather low. Immobilisation of the enzymes on Relizyme EP403/S in this case did not offer any appreciable improvement.

Table 3. Comparison of specific activities and expression levels of the original horse liver alcohol dehydrogenases (HLADH) and the two chimeric proteins in the free enzyme form. Specific activities of the resin following immobilisation of enzymes (1 mg_{enzyme}/g_{resin}) and percentage of residual activity after immobilisation (24 h, room temperature, in 50 mM phosphate buffer pH 8) with respect to the free protein.

	Free Enzyme		Immobilised Enzyme onto Sepabeads EC-EP/S (Pore ø 10–20 nm)		Immobilised Enzyme onto ReliZyme EP403/S (Pore ø 20–40 nm)	
	Specific Activity (U/mg)	Expression (mg/L)	Specific Activity (U/g)	Rescued Activity (%)	Specific Activity (U/g)	Rescued Activity (%)
HLADH	2.6 ± 0.3	20	0.80 ± 0.08	30.8	1.11 ± 0.05	32.4
T4L-HLADH_L1	0.18 ± 0.01	>1	0.08 ± 0.01	48.1	0.07 ± 0.02	38.9
T4L-HLADH_L2	0.15 ± 0.01	>1	0.07 ± 0.02	45.7	0.06 ± 0.01	39.9

The original HLADH lost about two-thirds of the activity, while the two chimeric enzymes responded better to the immobilisation, reaching around 50% of recovered activity. The stability of the immobilised enzymes was excellent for all the constructs under storage condition (4 °C in Tris-HCl buffer pH 8), with almost unchanged activity for over a month.

3. Discussion

This novel strategy of immobilisation, developed by introducing an inert protein linker, resulted in significantly enhanced recovered activity of the anchored enzymes. All the cases studied—HEWT, BS2m, and HLADH—when immobilised via T4L linking, showed an improvement in their retained activity, with a maximum of 90% displayed by the HEWT with the longer peptide linker. The most impressive result was achieved with the most challenging enzyme, BS2m esterase, which reached the performance of a competitive solid catalyst, with a maximum activity of 6.2 U/g when immobilised on Relizyme EP403/S. These results are superior to any other covalent method reported in the literature to date and might give an indication of the protective action that the protein spacer exercises by preventing direct bonding to the carrier. However, the expression levels and activity of the chimeras are not easily predictable and the immobilised enzymes may become more sensible to the reaction environment, partially diminishing the benefits of the immobilisation. Again, this was not observed for the chimeric BS2m which showed operational stability comparable to the commercial NovoZyme 435 and a recovered activity 8-to-10-fold higher than achievable with the BS2m control enzyme. While additional research will still have to be carried out to develop a universal method for successful immobilisation, this work shows that protein linkers should be considered in covalent strategies to maximise the activity of the solid catalyst. Further investigation of the composition and length of the peptide linker may also lead to more active constructs and, likewise, positioning of the T4L and His-tag at the C-terminus may be beneficial for enzymes where the active site is structurally affected by the N-terminus region.

4. Materials and Methods

4.1. T4L- HEWT, T4L-BS2m, and T4L-HLADH Constructs Generation

The pVL1392 vectors harbouring the T4-Lysozyme (T4L) was first used as templates for PCR amplification. The genes were amplified by PCR using Q5 High-Fidelity DNA Polymerase (New England Biolab). The primers FWD-T4L (5′-AAAAGGATCCGAACATCTTCGAGATGCTGCGCAT CGACGAAG-3′) and RVS-T4L (5′-AAAACTCGAGGTAAGCGTCCCAAGTTCCAGTACGGAAG GTAGTG-3′) were designed to incorporate *Bam*HI and *Xho*I restriction sites, respectively (underlined). The gel-purified PCR products were digested with *Bam*HI and *Xho*I and the genes were cloned into a *Bam*HI/*Xho*I digested pRSETb vector and ligated overnight with T4 ligase at 16 °C. The resulting clone, named pRSETb-T4L, was propagated in electrocompetent *E. coli* XL10-Gold cells and the construct was confirmed by sequencing analysis. The pRSETb-T4L D11N mutation was performed using the QuikChange Lightning Multi single point mutation kit (Agilent Technologies®,

Santa Clara, CA, USA) (primer: 5′-GCTGCGCATCGACCAGGGCCTGCGTCTCA-3′). The results were confirmed by DNA sequencing.

The pHESPUC plasmid was used as template for the HEWT cloning. The genes were amplified by PCR using Q5 High-Fidelity DNA Polymerase (New England Biolab, Ipswich, MA, USA). The oligonucleotide primers FWD-HEWT (5′-AAAACCATGGGATGCAAACCCAAGACTATCAGG CCCTGGACCGC-3′) and RVS-HEWT (5′-AAAAAAGCTTTCATGCGGTTGGCTCCTCTTGCGTGT GC-3′) were designed to incorporate *Nco*I and *Hind*III restriction sites, respectively (underlined). The gel-purified PCR products were digested with *Nco*I and *Hind*III and the genes were cloned into a *Nco*I/*Hind*III digested pRSETb-T4L vector and ligated overnight with T4 ligase at 16 °C. The resulting clone, named pRSETb-T4L-HEWT_L1 (link one), was propagated in electrocompetent *E. coli* XL10-Gold cells and the construct was confirmed by sequencing analysis.

In order to change the linker length, the XhoI restriction site after the T4L gene was mutated and substituted with a second NcoI site by directed mutagenesis with QuikChange Lightning Multi single point mutation kit (Agilent Technologies®) exploiting an oligonucleotide (5′-AACTTGGGACGCTT ACCTCCATGGCTGCAGCTGGTACCATGGG-3′) synthesised from Eurofins Genomics®. The construct pRSETb-T4L-HEWT_L1 was digested with NcoI, purified from agarose gel, and self-ligated overnight with T4 ligase at 16 °C. The resulting clone, named pRSETb-T4L-HEWT_L2 (link two), was propagated in electrocompetent *E. coli* XL10-Gold cells and the construct was confirmed by sequencing analysis.

The pET28b_BS2m and pEqADH plasmid were used as template for the BS2m and HLADH cloning. The genes were amplified by PCR using Q5 High-Fidelity DNA Polymerase (New England Biolab). The oligonucleotide primers FWD-BS2m (5′-GAAAACCATGGATGACTCATCAAATA GTAACGACTCA-3′), RVS-BS2m (5′-GAAAAAAGCTTTCAGGATCCTTCTCCTTTTGAAGGG AATAGCT-3′), FWD-HLADH (5′-GAAAACCATGGGATGAGCACAGCAGGAAAAGTAATA AAATG-3′), and RVS-HLADH (5′-GAAAAAAGCTTTTCAAAACGTCAGGATGGTACGGATAC-3′) were designed to incorporate *Nco*I and *Hind*III restriction sites, respectively (underlined). The gel-purified PCR products were digested with *Nco*I and *Hind*III and the genes were cloned into a *Nco*I/*Hind*III digested and gel-purified pRSETb-T4L-HEWT_L1 or pRSETb-T4L-HEWT_L2 vectors and ligated overnight with T4 ligase at 16 °C. The resulting clones, named pRSETb-T4L-BS2m_L1 and pRSETb-T4L-HLADH_L1 (link one), and pRSETb-T4L-BS2m_L2 and pRSETb-T4L-HLADH_L2 (link two), were propagated in electrocompetent *E. coli* XL10-Gold cells and the construct was confirmed by sequencing analysis.

4.2. Expression, Purification, and Characterization of the HEWT, BS2m, HLADH, and T4L Proteins in E. coli

Protein expression, purification, characterization of the original HEWT, BS2m, and HLADH, and their T4L constructs was performed following previously reported protocols [17,20,21]. The assay for the BS2m esterase (and NovoZyme 435) was adapted using the para-nitrophenyl acetate (PNPA) as standard substrate.

Protein quantification was determined by UV reading (280 nm) considering for each enzyme the calculated molar extinction coefficient (Table S1).

4.3. Immobilisation of HEWT, BS2m, and HLADH

The immobilisation of HEWT and HLADH were performed following the published procedures [18,28] originally developed by Guisan and co-workers [16]. For the BS2m immobilisation, 1 g of resin (Sepabeads® EC-EP/S or ReliZyme EP403/S from Resindion S.r.l., Milan, Italy) immobilisation support was treated with 2 mL of modification buffer (0.1 M of sodium borate and 2 M of iminodiacetic acid in phosphate buffer 50 mM pH 8.5) under gentle shaking for two hours at room temperature. The sample was then filtered and washed with distilled water and incubated with a metal containing solution (1 M of sodium chloride and 5 mg/mL of metal cation in phosphate buffer 50 mM pH 6) for two hours. 2 mL of enzyme solution, suitably prepared to provide a ratio

of 5 mg of enzyme per 1 g of beads, were added to the resin and kept under gentle agitation (2–24 h, 200 rpm orbital shaking, at room temperature). The beads were filtered and washed thoroughly with desorption buffer (0.05 M of EDTA and 0.5 M of sodium chloride in phosphate buffer pH 7.4 20 mM) and distilled water, 4 mL of blocking buffer (3 M of glycine in phosphate buffer pH 8.5 50 mM) were added and the suspension left under agitation for 20 h. Finally, the beads were washed, collected, and conserved in 2 mL of storage buffer (50 mM phosphate pH 8.0). The imm-BS2m was routinely stored at 4 °C. The activity of immobilised BS2m was determined by weighing an appropriate amount of resin (5–50 mg) into a 25 mL reaction tube with cap, followed by the addition of 10 mL reaction mixture (50 mM phosphate pH 8.0, containing 0.5 mM PNPA). The immobilised enzyme reaction mixture was shaken at 25 °C, 250 rpm and the absorbance at 410 nm was recorded every minute as single readings using Brand UV-cuvettes. The imm-BS2m specific activity (U/g) is defined as µmol of *para*-nitrophenol formed (ε = 15 mM^{-1} cm^{-1}) *per* minute *per* gram of immobilised enzyme.

Unless differently stated, chemicals were purchased from Sigma Aldrich Merck (St. Louis, MO, USA).

Supplementary Materials: The following are available online at www.mdpi.com/2073-4344/8/1/40/s1, Figure S1: SDS-gel (12%) electrophoresis of T4L-HEWTs, Figure S2: HEWTs immobilisation at different incubation times, Figure S3: Reusability profile of the immobilised T4L-HEWT_L1 (circle) and T4L-HEWT_L2 (square) after 10 reaction cycles, Figure S4: Stability of HEWTs in different organic co-solvent, Table S1: computed molecular weight (MW) and molar extinction coefficients (ε), Figure S5: SDS-gel (12%) electrophoresis of T4L-BS2ms, Figure S6: SDS-gel (12%) electrophoresis of T4L-HLADHs.

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Author Contributions: M.P. and F.P. designed the study. M.P. cloned, immobilised, and evaluated the HEWT and HLADH originals enzyme and T4L-variants. D.R.P. and M.L.C. cloned, immobilised, and evaluated the BS2m original enzyme and T4L-variants. M.P. and F.P. wrote the paper. All authors discussed the results and commented on the manuscript.

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