Immobilization of γ-Glutamyl Transpeptidase from Equine Kidney for the Synthesis of \textit{kokumi} Compounds

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γ-Glutamyl transpeptidase from equine kidney (ekGGT, E.C. 2.3.2.2) is an intrinsic membrane enzyme which transfers the γ-glutamyl moiety of glutathione to amino acids and peptides, thus producing γ-glutamyl derivatives. An immobilization study of ekGGT was carried out with the aim to develop a robust biocatalyst for the synthesis of γ-glutamyl amino acids which are known as \textit{kokumi} compounds. Heterofunctional octyl-glyoxy-l-agarose resulted in a high immobilization yield and activity recovery (93 % and 88 %, respectively). Immobilized ekGGT retained more than 95 % activity under reaction conditions (Tris-HCl, pH 9, 0.05 M) after 6 days, whereas the residual activity after 6 reaction cycles (18 days) was 85 %. The synthesis of γ-glutamylmethionine catalyzed by octyl-glyoxy-l-agarose-ekGGT afforded the product in 42 % yield (101 mg). The immobilized ekGGT was characterized by Raman spectroscopy. The immobilization protocol developed for ekGGT could be of general applicability to membrane proteins.

1. Introduction

The use of enzymes as biocatalysts in food, pharmaceutical and fine chemical industries has expanded significantly over the last decades. For a successful application of enzymes, these catalysts must be stable and fully functional under process conditions, which often differ from the cellular environment (temperature, presence of organic cosolvents, pH). Enzyme immobilization on solid supports is a widely used technique to stabilize proteins and improve their performance in synthetic applications. As a consequence of an enhanced stability, immobilization allows for enzyme reusability over multiple catalytic cycles. Moreover, once the biocatalyst is bound to the support, it becomes a heterogenous catalyst, thus facilitating its separation from the reaction mixture and minimizing the risk of product contamination. Protein impurities associated with molecules synthesized by biocatalysis represent an issue in food and pharmaceutical processes. Many immobilization techniques have been developed, including absorption, entrapment, encapsulation, cross-linking and covalent binding. The optimal immobilization method has to be determined experimentally for each enzyme as it strongly depends on the biocatalytic process as well as on the enzyme itself. Most of immobilization protocols reported in literature are applicable to water-soluble enzymes but to a very less extent to membrane-bound proteins, which require a quite hydrophobic environment to preserve their catalytic activity.\footnote{These two authors contributed equally to this work.}

Integral membrane proteins (IMPs) represent 15–30 % of all coding sequences in the genome of living organisms. IMPs play essential roles in living cells that can be exploited also \textit{in vivo} for biosensors, biocatalytic applications and energy production. The immobilization of membrane-bound proteins and the design of solid supports mimicking natural membranes are an active field of research. Liposomes, sol-gel materials and lipid crystalline cubic phases are some of the membrane mimetic-matrixes developed for IMP immobilization. By contrast, few examples of covalent immobilization have been reported to date. γ-Glutamyl-transpeptidases (GGTs, EC 2.3.2.2) catalyze the transfer of the γ-glutamyl moiety of glutathione (GSH) and other γ-glutamyl compounds to either water (hydrolysis) or natural and modified amino acids and peptides (transpeptidation), thus producing γ-glutamyl derivatives which are widely distributed in all living organisms from bacteria to mammals. Bacterial homologues are generally non glycosylated soluble proteins, which are localized in the periplasmic space or...
extracellular space, while mammalian GGTs are generally glycosylated IMPs.\textsuperscript{[29]}

GGTs have been exploited as biocatalysts for the synthesis of various \( \gamma \)-glutamyl compounds with high added value such as \( \gamma \)-L-glutamyl-L-DOPA, \( \gamma \)-glutamyl-taurine, \( \gamma \)-D-glutamyl-L-tryptophan and \( \gamma \)-L-glutamyl-ethylamide (L-theanine).\textsuperscript{[30]} Moreover, \( \gamma \)-glutamyl derivatives of methionine and S-substituted alk(en)yl cysteines (\textit{i.e.} methionylecysteine, S-allylcysteine and S-trans-propenylcysteine) are known as \textit{kokumi} compounds. \textit{Kokumi} is a Japanese term that refers to taste perception defined as having mouthfulness, thickness and a long-lasting savory sensation. Although being nearly tasteless in themselves, \textit{kokumi} compounds are able to elicit strong taste sensations, especially when associated with protein-rich food, thus acting as true flavor enhancers.\textsuperscript{[31,32]} Despite their simple chemical structure, the synthesis of \( \gamma \)-glutamyl derivatives through the classical peptide chemistry is troublesome due to the need of protection and deprotection steps.\textsuperscript{[33]} Therefore, an enzymatic approach does represent an appealing solution for their supply.

In this work, we described the immobilization study of equine kidney GGT (ekGGT) for the synthesis of \textit{kokumi} peptides. A tailor-made immobilization protocol was set up by considering that ekGGT is a membrane enzyme and that it is potentially usable as biocatalyst in food sector. The heterofunctional support octyl-glyoxyl (OCGLX)-agarose\textsuperscript{[34]} allowed for the immobilization of ekGGT in two steps: first, the hydrophobic core of the enzyme was absorbed and stabilized by the hydrophobic octyl chains of the support, thus mimicking lipids of cellular membranes, then, the covalent attachment of the enzyme through the aldehyde groups of the carrier stabilized the protein against potential enzyme leaching. Raman spectroscopy was used to characterize the molecular properties of native and immobilized ekGGT. Finally, the immobilized ekGGT was used for the preparative synthesis of the \textit{kokumi} substance \( \gamma \)-glutamylmethionine.

### 2. Results and Discussion

#### 2.1. Screening of Immobilization Carriers

With the aim to develop a ekGGT-based biocatalyst suitable for preparative applications, this enzyme was immobilized on differently activated agarose carriers. Agarose beads, derived from a neutral gelling heteropolysaccharide, are porous, mechanically resistant, and highly hydrophilic. These features make this biopolymer an ideal carrier for enzyme immobilization.\textsuperscript{[35]}

As a first approach, GLX-agarose (see Table 1 for abbreviations) was assayed. This carrier has been widely explored for the covalent immobilization of enzymes.\textsuperscript{[36–38]}

Immobilization relies on the formation of imine bonds between the aldehyde groups of the carrier and the lysine \( \varepsilon \)-NH\(_2\) groups of the protein. The immobilization of ekGGT on GLX-agarose (Table 1, entry 1) gave a high yield in terms of immobilized protein (80\%) and a good activity recovery (30\%).

Agarose is a versatile carrier since it can be easily functionalized by exploiting the reactivity of its hydroxyl groups. Thus, we decided to explore an array of binding chemistry. The reactive groups of GA-EDA-agarose (Table 1, entry 2) are the same as in GLX-agarose. On the same binding chemistry, however, GA-EDA-agarose is characterized by a spacer that may impart a higher flexibility to the immobilized enzyme. In principle, this scenario could assist in minimizing the distortion of the protein upon immobilization, thus preserving the enzyme activity.\textsuperscript{[39]} The immobilization yield was 100\% but, surprisingly,
the immobilized enzyme lost almost completely its activity (3% activity recovery). An even poorer outcome was obtained by using GA-APTES-agarose. When eGGT was immobilized on an epoxy carrier (GPTS-agarose), a moderate immobilization yield was achieved (37%), but no activity was detected for the immobilized biocatalyst. A slight improvement was obtained when the enzyme was immobilized on Sepabeads™ EC-EP/S (100% immobilization yield, 18% activity recovery), an epoxy-functionalized carrier which is hydrophobic in nature (methacrylic polymeric matrix). This evidence highlighted that, on the same binding chemistry, a less hydrophilic carrier might be better tolerated by the enzyme. In order to enlarge the screening to a different binding chemistry, carbamate and isocyanate activation of the carrier was performed by treatment of agarose with N,N′-carboxymethylidazole (CDI) or CNBr, respectively. In the former case, a scarce immobilization yield and a negligible activity recovery were obtained (Table 1, entry 5), whereas CNBr-agarose gave 85% immobilization yield and 41% activity recovery (Table 1, entry 6).

The use of heterofunctional supports, that bear different types of active groups on the support surface, has been previously reported for the immobilization of three lipases (from Candida antarctica B, Thermomyces lanuginosus and Rhizomucor miehei), and the artificial phospholipase Lecitase Ultra. Specifically, OC-agarose was converted into OCGLX-agarose. Cross-linked octyl-agarose beads have some diols (resulting from the opening of the epoxy moieties during carrier preparation) which may be easily oxidized to glyoxyxl groups with periodate.

We hypothesized that such a heterofunctional carrier might enhance the immobilization of eGGT through a first mild, hydrophobic interaction involving the hydrophobic portion of the enzyme and the octyl chains (mimicking the natural environment of the protein), followed by a covalent interaction enzyme-carrier deriving from the formation of imine bonds. This two-step mechanism can thus answer the need to create a "favourable" microenvironment for eGGT immobilization that prevents it from deactivation, also avoiding undesired enzyme leaching. Hydrophobic adsorption was carried out in phosphate buffer at pH 7 and, after 3 hours, a 98% immobilization yield was obtained. Then, once eGGT was adsorbed in a close proximity on the carrier surface, it was incubated at pH 10 to promote the reactivity of lysine ε-NH₂ groups towards the aldehyde groups of the carrier (Scheme 1). As a result, immobilization of eGGT was almost complete (93%) and a very high immobilization efficiency was obtained (95%). In other words, all the enzyme in solution was immobilized and almost 100% of activity was found in the immobilized biocatalyst.

### 2.2. Study of the Immobilization Process of eGGT on OCGLX-Agarose

A systematic study was undertaken in order to evaluate the critical factors that affected the immobilization of eGGT on OCGLX-agarose. The goal of this study was to set up a robust and reproducible method for this immobilization protocol by using an experimental design approach (DoE). Upon the critical revision of all the immobilization steps, six factors (storage time of the carrier X1, buffer volume X2, stirring system X3, NaBH₄ addition X4, time of chemical reduction X5, drying time of the immobilized biocatalyst X6) were selected. One additional "Dummy" fictitious factor, used to assay the experimental variance, was included in the list of the process factors, as shown in the experimental plan reported in Table 2.

Eight experiments were thus planned by following a Plackett Burman 8-run design (Table 2), and one further independent validation experiment was carried out to confirm the results. The model hypothesized for the description of the immobilization outcome was a linear model without interactions, as from the general Equation (1):

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y (activity \text{ U/g}) = b_0 + b_1 \cdot X_1 + b_2 \cdot X_2 + b_3 \cdot X_3 + b_4 \cdot X_4 + b_5 \cdot X_5 + b_6 \cdot X_6 + b_7 \cdot X_7
\]

(1)

Three factors, namely the storage time of the carrier, the time of chemical reduction with NaBH₄, and the drying time of the immobilized biocatalyst (X1, X5, and X6) resulted to be those affecting more than the others the immobilization outcome. As shown in Table 2 and Figure 1, the carrier storage at 4°C (X1) was the most important factor. The activity of the immobilized enzyme was almost completely lost when the immobilization protocol was performed after 7 days from carrier preparation. The immobilization has to be carried out, indeed, after a maximum lag time of one day after the carrier preparation.

The second factor affecting the immobilization outcome was the time necessary for the chemical reduction with NaBH₄: the longer is the time of contact enzyme-reducing agent, the lower is the activity of immobilized eGGT. Unspecific reduction of amino acid residues of the protein altering the three-
The OCGLX-agarose-ekGGT was further characterized by Raman spectroscopy for a deeper insight into the binding of the enzyme to the carrier in label-free through a direct, non-destructive approach.

The analysis was performed on the carrier as such, on the native enzyme, and on the immobilized enzyme by acquiring the single spectra. As shown in Figure 2A, both the enzyme and the carrier were characterized by specific Raman signals.\(^{[40]}\) In the enzyme spectrum (Figure 2A), vibrations associated to phenylalanine (aromatic stretching, 1002 cm\(^{-1}\)), peptide bonds (amide C–N, N–H, 1305 cm\(^{-1}\)), aliphatic chains of amino acids (CH\(_2\), 1447 cm\(^{-1}\)) and amino acids backbone (C–O, 1631 cm\(^{-1}\)) have been detected, while in OCGLX-agarose spectrum typical signals related to mono- and disaccharides (C–O–C, 848, C–C, 891, C–O, 1083 cm\(^{-1}\)) and signals that can be associated to the aliphatic chain (CH\(_2\), 1454 cm\(^{-1}\)) are present. In the spectrum of the immobilized biocatalyst (Figure 2A), Raman signals of both ekGGT and OCGLX-agarose are consistent with the occurrence of an enzyme-carrier interaction (as highlighted by the shift of some Raman signals: 891, 964, 1077 cm\(^{-1}\)).

Once the reference spectra of the two standards (i.e. native enzyme and carrier) were obtained and after the analysis of the spectrum of the immobilized biocatalyst, a Raman imaging mapping experiment was performed in order to explore a small portion (around 1.5-2 µm of agarose microspheres) of the enzyme/OCGLX-agarose conjugate. As shown in Figure 2B, the surface of agarose beads is completely surrounded by the enzyme, thus suggesting a binding between the enzyme and support.

### 2.4. Stability of Immobilized and Native ekGGT

The stability of native ekGGT and OCGLX-agarose-ekGGT were evaluated under reaction conditions (Tris-HCl buffer, pH 9, 0.05 M 4 °C, in absence of substrate). The immobilized ekGGT was fully stable for 6 days, while the native enzyme retained 70% of its activity under the same incubation time (Figure 3).

Protein leaching assay on immobilized ekGGT was also performed. After 6 day-incubation under the reaction conditions, no release of the protein from the carrier was detected, as indicated by the absence of protein in the supernatant (see Experimental Section for details).

### 2.5. Immobilized Biocatalyst Recycling

Recycling of immobilized ekGGT was performed by evaluating over time the residual activity of the enzyme in the synthesis of...
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γ-glutamylmethionine in Tris-HCl buffer (pH 9, 0.05 M). After each reaction cycle (3 days), the reaction mixture was filtered under reduced pressure and the immobilized biocatalyst was re-used for the following reaction runs. As depicted in Figure 4, the immobilized eKGGT was successfully re-used for 6 cycles (18 days) and retained about 80% of its activity, thus showing an optimal recyclability. This result suggests that OCGLX-agarose-eKGGT could be re-used for additional reactions. Furthermore, it is worth pointing out that immobilization applied to eKGGT exerted more than the “usual” advantages derived from the heterogeneous catalysis (i.e. recyclability, re-use, protein stabilization, control of the process). In fact, eKGGT is a paradigmatic example of how immobilization can be also a viable technique to offset the high costs associated with production and use of expensive and hardly available enzymes (eKGGT is not a recombinant protein but it is extracted from natural sources in a low yield).

2.6. Preparative Synthesis of γ-Glutamylmethionine

In order to demonstrate the feasibility of a true biocatalytic application with the immobilized eKGGT, OCGLX-agarose-eKGGT was used to catalyze the synthesis of γ-glutamylmethionine also at a preparative scale. 5 U of OCGLX-agarose-eKGGT were added to a L-methionine solution (333 mM) in Tris-HCl buffer (pH 9, 0.05 M) in the presence of a 10% molar excess GSH. After 72 hours, the reaction product was isolated by preparative ion exchange chromatography in 42% yield. This result was consistent with the data obtained by using the non-immobilized enzyme.
3. Conclusions

In this work, we have developed a tailor-made immobilization for ekGGT, a membrane enzyme which can be used for the synthesis of γ-glutamyl amino acids having a great interest for food applications. γ-Glutamylmethionine was used as the reference kokumi compound to demonstrate the feasibility of the ekGGT-catalyzed synthetic application and the excellent recyclability/stability of the biocatalyst. The heterofunctional carrier OCGLX-agarose conveys hydrophobic alkyl chains and aldehyde groups which result in a concurrent non-covalent and covalent immobilization of the protein. On one hand, enzyme-carrier hydrophobic interactions, mimicking what occurs in vivo when the enzyme is anchored to the lipid membrane, are assumed to stabilize the tertiary folded structure of the protein, on the other hand, the formation of stable C–N bonds between the enzyme and the carrier further stabilizes the protein which can be thus used also in non-conventional environments.

A key issue in developing an immobilized enzyme for biocatalytic applications also includes a strict control of all the numerous variables associated with the immobilization protocol. In this frame, the Plackett-Burmann design has clearly highlighted which were the critical factors and the corrections needed in order to set up a robust and reproducible immobilization procedure.

Experimental Section

γ-Glutamyl transpeptidase from equine kidney (ekGGT), octyl-Sepharose® CL-4B (octyl-agarose, OC-agarose), L-glutamic acid γ-(4-nitroanilide) (GpNA), glycylglycine (Gly-Gly), 4-nitroaniline, L-methionine, glutathione (GSH), Bradford reagent, cyanogen (4-nitroanilide) (GpNA), glycylglycine (Gly-Gly), 4-nitroaniline, L-methionine, potassium phosphate, N,N’-carbonyldiimida-

zole (CDI), Dowex 1 x 8 ion exchange resin pad (200–400 mesh), ninhydrin reagent were purchased from Sigma Aldrich (Milano, Italy). Analytical thin layer chromatography (TLC) was performed on silica gel F254 precoated aluminium sheets (0.2 mm layer, Merck, Darmstadt, Germany). SepharoseTM 6B-84 (agarose) was from Amersham Biosciences (Uppsala, Sweden). Sepabeadxtm EC EP/S was a gift of Resindion S.r.l (Binasco, Italy). Sodium bicarbonate was from Carlo Erba (Cornaredo, Italy). Spectrophotometric assays were performed using a Shimadzu UV-1601 UV-Visible spectrophotometer equipped with magnetic stirring. Raman and UV-Vis spectroscopy was performed with a Aramis Horiba Jobin-Yvon micro-Raman spectrometer equipped with solid state lasers operating at 633 nm and 785 nm and with a DuoScan mapping mode configuration.

ekGGT Activity Assay

The standard activity assay (2 mL) was performed at room temperature in Tris-HCl buffer (pH 8.5, 0.1 M) containing GpNA (0.001 M), Gly-Gly (0.1 M) and an appropriate amount of enzyme (free ekGGT: 1 μg; immobilized ekGGT: 5–15 mg, under magnetic stirring). The reaction was monitored spectrophotometrically by measuring the formation of 4-nitroaniline at 410 nm in kinetic mode. The amount of 4-nitroaniline produced by the enzyme was quantified by using a calibration curve and an extinction coefficient of 8300 M−1 cm−1. One unit of ekGGT was defined as the amount of enzyme that produces 1 μmole of 4-nitroaniline per minute from GpNA in the presence of the acceptor Gly-Gly. The protein content in the solid crude extract of ekGGT was 50% of its weight based on Bradford assay; all the activity data reported in this paper are referred to this percentage.

Preparation of Agarose based Carriers

Glyoxyl-agarose (GLX-agarose) was prepared as reported in literature. Briefly, SepharoseTM 6B (agarose, 5 g) was suspended in dH2O (1.4 mL) and NaOH (1.7 M, 2.4 mL) containing NaBH4 (28.4 mg mL−1). Subsequently, glycidol (1.7 mL) was added dropwise keeping the vessel at 4 °C for 3 hours. Oxidation was kept under gently stirring overnight at 25 °C. After the incubation period, the suspension was filtered and the carrier was washed abundantly with dH2O. Oxidation was initiated by adding NaIO4 (0.1 M, 34 mL). The reaction was carried out for 2 hours at room temperature, then the carrier was filtered under reduced pressure and washed abundantly with dH2O and stored at 4 °C.

GA-EDA-agarose was prepared as described in literature. GLX-agarose (2 g) was aminated using EDA (pH 10, 2 M, 11.43 mL) for 2 hours and subsequently reduced for 2 hours with NaBH4 (11.4 mg). The EDA-activated agarose was then suspended in KH2PO4 buffer (pH 7, 0.2 M, 2.3 mL) and a solution of GA (25% (v/v) in dH2O, 3.4 mL) was added. The mixture was kept under mechanical stirring for 16 hours at room temperature in the darkness. The activated carrier was washed abundantly with dH2O and stored at 4 °C.

GA-APTES-agarose was prepared by modifying a protocol described in literature. Briefly, SepharoseTM 6B (agarose, 3 g) was suspended in NaOH (0.5 M, 50 mL) for 2 hours. The carrier was filtered, washed with dH2O and 4°C acetone/water (1:1). 10% (v/v) in dH2O, 60 mL) for 24 hours. Then, the carrier was washed and suspended in KH2PO4 buffer (pH 7, 0.2 M, 10 mL) and GA (25% v/v in dH2O, 6 mL). The mixture was kept under stirring for 16 hours at room temperature in the darkness.

GPTS-agarose was prepared as described in literature. Briefly, dry agarose (3 g) was suspended in dry toluene (60 mL). Subsequently, GPTS (3 mL) and Et,N (0.45 mL) were added and the reaction was kept for 24 hours at room temperature under stirring. The resulting carrier was filtered, washed and dried at 50 °C for 3 hours.

CDI-agarose was prepared by modifying the protocol described in literature. Briefly, dry agarose (3 g) was suspended in dry acetonitrile (60 mL). Subsequently, GA (25% v/v) in dH2O, 60 mL) for 24 hours. Then, the carrier was washed and suspended in KH2PO4 buffer (pH 7, 0.2 M, 10 mL) and GA (25% v/v in dH2O, 6 mL). The mixture was kept under stirring for 16 hours at room temperature in the darkness. The activated carrier was filtered, washed and dried at 50°C for 3 hours.

Commercial cyanogen bromide-activated-Sepharose® 4B (CNBr-agarose) (1 g) was hydrated in HCl (0.001 M, 200 mL) for 30 minutes. Then it was washed using NaHCO3 buffer (pH 8, 0.1 M) and used for the immobilization procedure.

OCGLX-agarose was prepared by octyl-Sepharose® CL-4B (OC-agarose) oxidation as reported in literature. The glyceryl groups of OC-agarose (1 g) were oxidized using NaIO4 (35 μmol) for 2 hours at room temperature under stirring. The oxidation degree of the carrier was monitored spectrophotometrically by measuring the periodate assay of the supernatant. Following this procedure, the carrier
was functionalized with 25–30 μmol aldehyde groups per gram of carrier.

**ekGGT Immobilization**

For all the immobilization procedures, an enzyme loading of 1 mg was used per gram of carrier. A 10:1 ratio volume of immobilization reaction/volume of the carrier was used. The protein content in the solid crude extract of ekGGT was 50% of its weight based on Bradford assay.[42] During immobilization, the supernatant was monitored by measuring the amount of protein in solution (Bradford assay[42]), and the residual activity of the supernatant (20 μL) was checked by the standard activity assay described before.

Immobilization of ekGGT on GLX-agarose was performed following the standard protocol.[43] Briefly, GLX-agarose was washed abundantly with NaHCO$_3$ buffer (pH 10, 0.05 M) and then filtered under reduced pressure until dryness. The solid crude extract of ekGGT (400 μg, 200 μg of protein) were solubilized into NaHCO$_3$ buffer (2.52 mL). Then, the carrier (200 mg) was added and the suspension was allowed to stir for 4 hours at room temperature. Finally, NaBH$_4$ (2.5 mg, 250 μg of protein) was added to the mixture and incubated for 30 minutes for imino bonds reduction. The immobilized enzyme was then filtered, washed with Tris-HCl buffer (pH 8.5, 0.1 M) and stored at 4 °C.

The immobilization of ekGGT on GA-EDA-agarose and GA-APTES-agarose was carried out in NaHCO$_3$ buffer (pH 10, 0.05 M), while the immobilization on GPTS-agarose and CDI-agarose was performed in KH$_2$PO$_4$ buffer (pH 8, 0.05 M). The solid crude extract of ekGGT (500 μg, 250 μg of protein) was solubilized in the suitable buffer (3.15 mL) and added to the activated carrier (250 mg); the suspension was allowed to stir at room temperature. After 24 hours, the carrier was filtered under reduced pressure, washed with Tris-HCl buffer (pH 8.5, 0.1 M) and stored at 4 °C.

The immobilization of ekGGT on CNBr-agarose was performed following a standard protocol.[30] ekGGT solid crude extract (540 μg, 270 μg of protein) was dissolved in NaHCO$_3$ buffer (pH 8, 0.1 M, 3.5 mL). Subsequently, the hydrated carrier (270 mg), prepared as previously described, was added. After 3 hours, the immobilized biocatalyst was filtered and suspended in Tris-HCl buffer (pH 8, 0.1 M, 3.5 mL) for 2 hours. Then, the immobilized biocatalyst was washed with Tris-HCl buffer (pH 8.5, 0.1 M) and stored at 4 °C.

The immobilization protocol on OCGXL-agarose was carried out in two steps: first, the protein was adsorbed to the carrier, then the reaction between the carrier aldehydes and the enzyme amino groups took place.[34] OCGXL-agarose was washed with KH$_2$PO$_4$ buffer (pH 7, 0.025 M). ekGGT solid crude extract (500 μg, 250 μg of protein) was solubilized in the same buffer (3.15 mL) in the presence of the carrier (250 mg).[44] After 3 hours, all the protein in the solution was absorbed onto the carrier; the biocatalyst was filtered and suspended in NaHCO$_3$ buffer (pH 10, 0.05 M, 3.15 mL). Immobilization was carried on for further 3 hours and then NaBH$_4$ (3 mg) was added. After stirring for 30 minutes, the biocatalyst was washed with Tris-HCl buffer (pH 8.5, 0.1 M) and stored at 4 °C. For the preparative synthesis of γ-glutamylmethionine the protein loading was scaled-up to 2 mgg$^{-1}$.

Immobilization on Sepabeads$^{TM}$ EC-EP/S was performed as reported in literature.[45,46] Commercial Sepabeads$^{TM}$ EC-EP/S was hydrated with dH$_2$O for 1 hour under mechanical stirring. Then, the carrier was washed with KH$_2$PO$_4$ buffer (pH 8, 1 M). ekGGT solid crude extract (500 μg, 250 μg of protein) was solubilized in the same buffer (3.15 mL) and the carrier (250 mg) was added to the solution. After 24 hours, the carrier was filtered and resuspended in KH$_2$PO$_4$ buffer (pH 8, 1 M, 3.15 mL) containing glycine (3 M) for the quenching step. After 21 hours, the immobilized biocatalyst was washed with Tris-HCl buffer (pH 8.5, 0.1 M) and stored at 4 °C.

**Plackett-Burman Design**

The first experiments of immobilization were performed using freshly prepared OCGXL-agarose (one day before starting the experiments). On the day one, 4 experiments were carried out (reactions 2, 3, 5 and 8, Table 2). Specifically, ekGGT (1.6 mg) was dissolved in KH$_2$PO$_4$ buffer (pH 7, 0.025 M, 0.8 mL); meanwhile the carrier was conditioned with the same buffer and 200 mg of OCGXL-agarose were used for each immobilization trial. Phosphate buffer (pH 7, 0.025 M) was added in a vessel (4 mL or 2 mL) for reactions 5 and 8, respectively, followed by the ekGGT solution (0.2 mL). Both the reactions were kept under mechanical stirring. The same procedure was carried out for reactions 2 and 3 (4 mL or 2 mL, respectively) but in this case, the reactions were stirred by a rotary shaker. After 3 hours, solid NaBH$_4$ (1 mg mL$^{-1}$) was added to reactions 5 and 8. After 30 min, reaction 8 was filtered and washed with Tris-HCl buffer (pH 8.5, 0.1 M) and dried for 3 min. The same procedure was performed for reaction 5, but prolonging to 60 min the chemical reduction step. In this case, the immobilized ekGGT was filtered and dried s.v. for 10 min. For reactions 2 and 3, NaBH$_4$ was dissolved in water (2 mg mL$^{-1}$) prior to addition to the reaction mixture (4 mL and 2 mL, respectively). After 30 min, reaction 2 was filtered, washed with Tris-HCl buffer (pH 8.5, 0.1 M) and dried for 10 min, whereas the reduction step for reaction 3 was 60 min and the drying time of the biocatalyst after filtration s.v. was 3 min. The standard activity assay was performed twice on each of the 4 immobilized biocatalysts.

The immobilization was also performed using OCGXL-agarose stored at 4 °C for one week. On the seventh day, 4 experiments were carried out (reactions 1, 4, 6 and 7, Table 2). Specifically, ekGGT (1.6 mg) was dissolved in KH$_2$PO$_4$ buffer (pH 7, 0.025 M, 0.8 mL); meanwhile the carrier was conditioned with the same buffer and 200 mg of OCGXL-agarose were used for each immobilization trial. Phosphate buffer (pH 7, 0.025 M) was added in a vessel (2 mL or 4 mL) for reactions 4, 6 and 1, 7, respectively, followed by the ekGGT solution (0.2 mL). Reactions 4 and 7 were kept under mechanical stirring while reactions 6 and 1 were stirred by a rotary shaker. After 3 hours, to reactions 4 and 7, an appropriate volume of an aqueous stock solution of NaBH$_4$ (2 mg mL$^{-1}$) was added in order to reach a final concentration of 1 mg mL$^{-1}$. After 30 min, reaction 7 was filtered, washed with Tris-HCl buffer (pH 8.5, 0.1 M) and dried for 3 min, whereas the reduction step for reaction 4 was 60 min and the drying time of the biocatalyst after filtration s.v. was 10 min. Reactions 1 and 6 were incubated with solid NaBH$_4$ (1 mg mL$^{-1}$) for 60 min and 30 min, respectively. After 21 hours, the chemical reduction step, reactions 1 and 6 were washed and dried s.v. for 3 and 10 min, respectively. The standard activity assay was performed twice on each of the 4 immobilized biocatalysts.

**Raman Spectroscopy**

The enzyme (ekGGT), the carrier (OCGLX-agarose), and the immobilized biocatalyst (OCGLX-agarose-ekGGT) were analyzed by a Raman microspectroscope equipped with a diode-pumped solid-state laser operating at 532 nm and a Peltier-cooled CCD detector. Each sample was deposited on a calcium fluoride slide and allowed to air dry. All the measurements were performed with 100× objective (NA 0.75, Olympus, Tokyo, Japan), 1800 grooves/mm diffraction grating, 400 μm entrance slit, and confocal mode.
were suspended in Tris-HCl buffer (pH 8.5, 0.05 M, ca 1 mL). The recycle of immobilized ekGGT was performed according to the procedure described in literature. One glass sample holder with a stirring bar was filled with L-methionine (149 mg, 1.0 mmol) and GSH (338 mg, 1.1 mmol). L-Methionine and GSH were solubilized in Tris-HCl buffer (pH 9, 0.05 M, 1 mL) under stirring. The pH was adjusted to 9 with NaOH (2 M) and stirring was maintained until the solution was completely clear. Afterwards, 2 mL of the same buffer were added to the reaction mixture. A specific amount of immobilized biocatalyst (5 U) was added. The mixture was kept at 4 °C under stirring for 3 days. The reaction mixture was filtered to remove the immobilized enzyme and the immobilized biocatalyst was re-used for the second reaction run. The residual activity of immobilized ekGGT was measured by the standard activity assay described before.

Preparative Synthesis of γ-Glutamylmethionine

L-Methionine (149 mg, 1.0 mmol) and GSH (338 mg, 1.1 mmol) were suspended in Tris-HCl buffer (pH 8.5, 0.05 M, ca 1 mL). The pH was adjusted to 9.0 with NaOH (2 M) until a clear solution was obtained, then the solution was diluted to a final volume of 3 mL with the same buffer. The reaction was initiated by addition of protein in the supernatant (Bradford assay) with a stirring bar was filled with L-methionine (149 mg, 1.0 mmol) and GSH (338 mg, 1.1 mmol). L-Methionine and GSH were solubilized in Tris-HCl buffer (pH 9, 0.05 M, 1 mL) under stirring. The pH was adjusted to 9 with NaOH (2 M) and stirring was maintained until the solution was completely clear. Afterwards, 2 mL of the same buffer were added to the reaction mixture. A specific amount of immobilized biocatalyst (5 U) was added. The mixture was kept at 4 °C under stirring for 3 days. The reaction mixture was filtered to remove the immobilized enzyme and the immobilized biocatalyst was re-used for the second reaction run. The residual activity of immobilized ekGGT was measured by the standard activity assay described before.

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Conflict of Interest

The authors declare no conflict of interest.

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