Biotechnology and Bioprocess Engineering 28: 379-385 (2023)
DOI 10.1007/s12257-022-0350-z

pISSN 1226-8372
eISSN 1976-3816

REVIEW PAPER

Impact of the Synthetic Scaffold Strategy on the Metabolic Pathway Engineering

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Received: 14 November 2022 / Revised: 7 March 2023 / Accepted: 13 March 2023

Abstract For the development of the efficient bio-refinery process or biochemical producer, metabolic engineering has become an attractive choice recently. However, engineered metabolic pathways often suffer from flux imbalances due to a lack of corresponding regulatory mechanisms associated with natural metabolism. The interaction among different enzymes within a metabolic pathway plays an important role in regulating the efficiency of metabolic processes. Consequently, the creation of protein scaffolds has helped with the spatial co-localization of proteins in metabolic engineering. Research on protein scaffolds indicated scaffold systems may enhance metabolic productivity further. In this review, the specificity, selectivity, and regulatory mechanisms of protein-protein interactions are discussed in the context of the important effects that they exert on various biological processes.

Keywords: scaffold protein, metabolic engineering, synthetic biology, *Escherichia coli*

1. Introduction

Producing fuel from renewable resources, particularly by converting biomass into specialty chemicals, has become an attractive research challenge [1]. Advanced technologies have enabled DNA synthesis, microbial genome sequencing, and extraction and redesign of systems in user-friendly hosts such as *Escherichia coli*. Such engineered bacterial strains have increased recombinant protein production [2]. However, enzymatic reaction's flux within biochemical pathways, particularly the breakdown of toxic intermediates, needs to be balanced to achieve high product titers.

The scaffold systems (DNA, RNA and protein) are multifunctional enzyme system mimicking natural systems, which presents a versatile approach in synthetic biology [3]. This technology involves designing and engineering DNA or RNA molecules that serve as scaffolds to bring enzymes and other biomolecules together in a specific spatial configuration. The use of scaffolds (DNA, RNA and protein) has several advantages over traditional methods of metabolic engineering, such as the use of synthetic protein scaffolds. DNA and RNA scaffolds can be designed and synthesized using simple molecular biology techniques, allowing for easy and flexible modification of their properties [4].

Scaffold proteins have been observed by their spatial proximity to enzymes and have critical roles in cellular signaling pathways [4]. The "scaffold" concept was created by building the module block (Fig. 1). Scaffold proteins engage multiple binding partners and facilitate their concerted interaction and function, such that they reduce the diffusion or competing pathways of biochemical intermediates, decrease their transit times, and avoid unfavorable kinetic equilibria resulting from metabolite concentrations in the bulk phase [5].

Several protein-protein interaction modules have been investigated in detail including PDZ (postsynaptic density 95/discs large/zona occludens-1), SH2, SH3 (Src homology), GTPase protein binding (GBD) domain [6], mitogen-activated protein kinase (MAPK) signaling during mating in the budding yeast, using the scaffold Ste5p [7], neuronal

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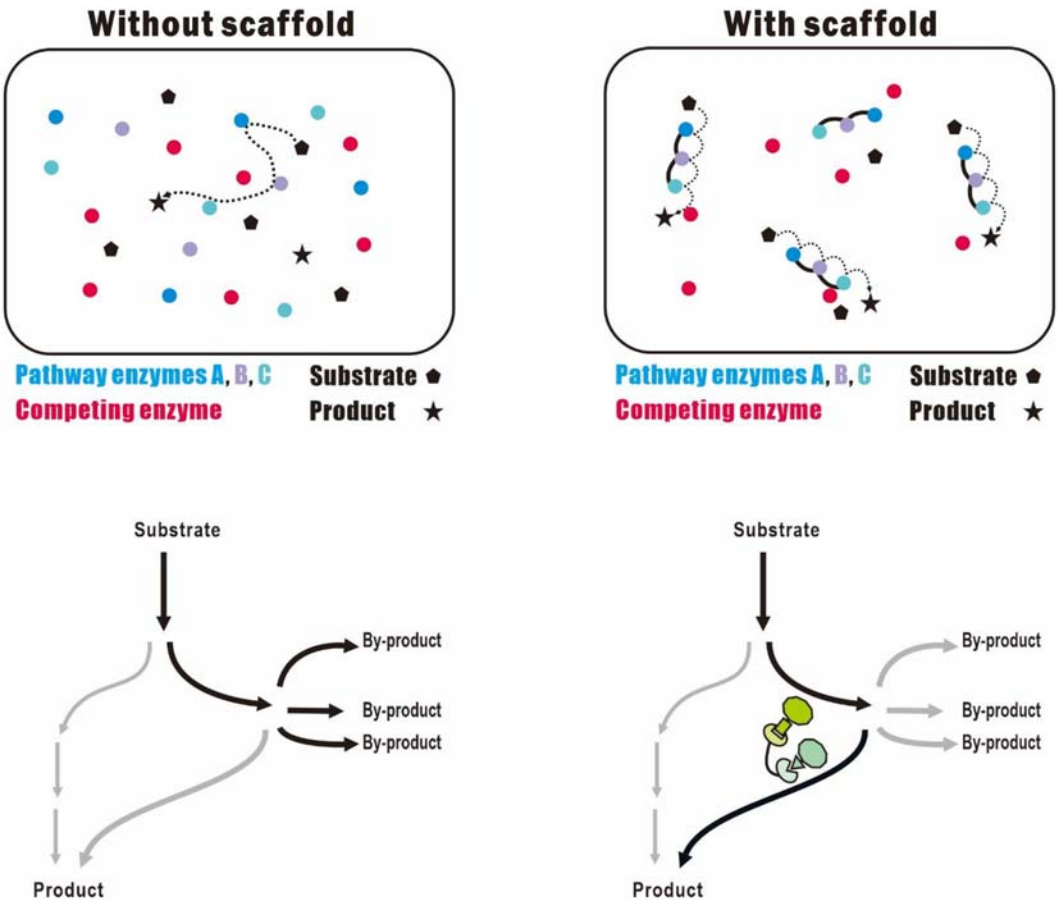

Fig. 1. A schematic drawing showing the mechanism of the synthetic scaffold in intracellular metabolism. By the introduction of the synthetic scaffold, the substrate can react with desired pathway enzymes, and metabolic flux can be derived to the desired product. Scaffold protein is leading to increased catalytic performance and product yield.

synaptic signaling exploiting PSD-95 [8], and photosensory reception in drosophila signaling using InaD [9].

2. Protein Scaffolds

In the target proteins, protein interaction modules are often described by short amino acid motifs at the C-termini. The most notable modules among these are PDZ, SH2, SH3 (Src homology), and GBD domains [10]. The concept is used often in research because of the advancement of peptide motifs and cognate adaptor domains. A variety of target metabolites, including catechin [11], D-glucaric acid [12], H_2 [13], resveratrol [14], butyrate [15], gamma-aminobutyric acid (GABA) [16], mevalonate [17], and itaconate [18], have been produced successfully by applying SH2, SH3, GBD, and PDZ domains. These proteins have a sequence motif, which is a small protein domain. The scaffold's binding nature differs based on the protein's physiological roles [19]. Protein interactions are termed short linear interaction motifs (SLiM) in those cases where the adaptor domain adopts a globular, three-dimensional structure. The shape of the scaffold is critically affected by three factors, including the domain, the linker, and the peptide ligand.

The concept of scaffolding proteins proposes the SLiM as the binding partner for protein adaptor domains. SLiMs usually encompass only 3–10 amino acids and possess several properties, which are suitable as synthetic biology ligands. Since they are short motifs, they often occur in disordered protein regions. These peptide motifs occur in 20–50% of all eukaryotic proteins, and 17% of these are muddled in eukaryotic cells. Around 300 such motif patterns are listed in the eukaryotic linear motif resources, including the ELM database [20], PROSITE [21], and Minimotif-Miner [22]. To ensure fitting spacing between the binding residues, SLiMs generally comprise variable residues such as the SLiM sequence patterns for binding to SH3 domains or a phosphorylated tyrosine residue, with a peculiar sequence nearby for binding to SH2 domains.

Adaptor domains must exhibit a strong affinity toward their specific peptide ligands. This feature allows defined paring even during the simultaneous usage of several domain-ligand pairs. Koch *et al*. [23] reported that the first mediate interaction with SLiMs is exhibited by the "Src homology 2" (SH2) and "Src homology 3" (SH3) domains. The SH3 domains are small modules of 60 amino acids with proline-rich ligands capable of binding to the surface of three hollow grooves domains. The aromatic residues are conserved and exhibit two different binding orientations [24]. The 100 amino acids are highly conserved structures of SH2 domains**,** comprised of two α-helices and seven β-strands, and contain an N-terminal catalytic domain [25]. Several SH3 and SH2 domains with different ligand binding specificities have been described in the last decade [26].

PDZ domains are similar in size to SH2 domains but contain specific motifs at the C-terminus of their binding partners. Over 200 PDZ domain structures have been reported so far [27]. Most peptide ligands adopt a β-strand conformation and extend an existing β-sheet within the PDZ domain upon binding [28]. The most recently reported protein-peptide scaffolding is the GBDs. In contrast to other scaffolds, under physiological conditions, the GBD domains do not exhibit a single adopter, but rather show multiple, loosely-packed conformations in solution [29,30]. SH2, SH3, PDZ, and GBD exhibit sufficiently high affinities and specificities for their ligand.

One of the important constituents of modular protein scaffolding is the linker. The protein construct's activity and folding properties could be affected by the linker's length and amino acid composition [31-33]. Among the two reported data guiding the artificial linkers' rational design, one presents a preferred mean linker length of 6.5 residues [34], while the second suggests this length is 10.0±5.8 residues [35]. The designed linkers may be classified and defined according to their functionality and structure [36].

The MAPK cascades are often used in all eukaryotic cells as a center for complex signal transduction pathways [37]. Some serially activated protein kinases are named by the last kinase of the series in every MAPK cascade within a module. Surprisingly, the identical MAPK cascade can perform different functions in response to different stimuli or different levels of the same stimulus [38]. A scaffold was introduced in the yeast *Saccharomyces cerevisiae* to increase the local concentration of proteins of the phosphorylation cascade to facilitate the functioning of this cascade [39]. Ste5p, a protein of unknown biochemical function, interacts with protein kinases that operate at each step of the MAPK pathway, namely, Fus3p (a MAPK), Ste7p (a MAPKK), and Ste11p (a MAPKKK) signal cascade involved in the mating of budding yeast [7]. Ste5p reportedly serves as a scaffold to connect the interactions among various members of the kinase cascade. This allows a more efficient signal propagation and attenuation and minimizes crosstalk with other MAPK cascades, which thus ensures the integrity of the pheromone response pathway.

3. Application to Metabolic Engineering

Metabolic engineering is an advantage in providing environmentally safe, using cheap, and special sources in the production of high-value compounds. Metabolic engineering requires complementary strategies to achieve desired production yields that are near the theoretical maximum, which is essential for industrial viability. Several technologies improve production, including modeling metabolic and cellular behavior [40], predictable control over gene expression [41,42], and directed evolutionary approaches to improve enzyme characteristics [43]. In this review, we focus on the impact of scaffolding proteins in metabolic engineering (Fig. 2).

In nature, for optimal metabolic pathway performance, numerous examples of enzymes forming complexes are observed. Such as tryptophan synthase, carbamoyl phosphate synthase, and glutamine phosphoribosyl pyrophosphate amidotransferase, which protected reactive intermediates from the bulk solution by tunnels connecting the catalytic site structure [44].

The primary consideration when introducing scaffolding within metabolic pathways choosing colocation components. Each enzyme is combined with a specific ligand which is specularly interacted with a protein-protein domain. This renders the protein-protein interaction domains structural modularity play an important role in controlling the binding activity in the non-native context of translational fusions. The SH3, PDZ, GBD, and leucine zipper families, tend to hold the binding activities of N- & C-terminal, or internal fusions and, given sufficient linker lengths, but not often required for the optimization of linkers to achieve the binding activity. Despite the robust binding activity, scaffold architecture, which is described by linker length and the number of protein-protein interaction domains, also influences overall flux improvement. Another important parameter is designing scaffolds in the low or lower micro-molar range of target enzymes. Protein-protein interaction domains are the members of families which have particularly attractive choices for scaffolding cause of a set of domains that potentially recognize specific ligands orthogonally. For example, the SH3 domains freely interact with other SH3 domain family ligands within the *S. cerevisiae* [45]. Thus, SH3 selection should reduce crosstalk and the number of orthogonal domain/ligand pairs available for simultaneous use is appreciably increased.

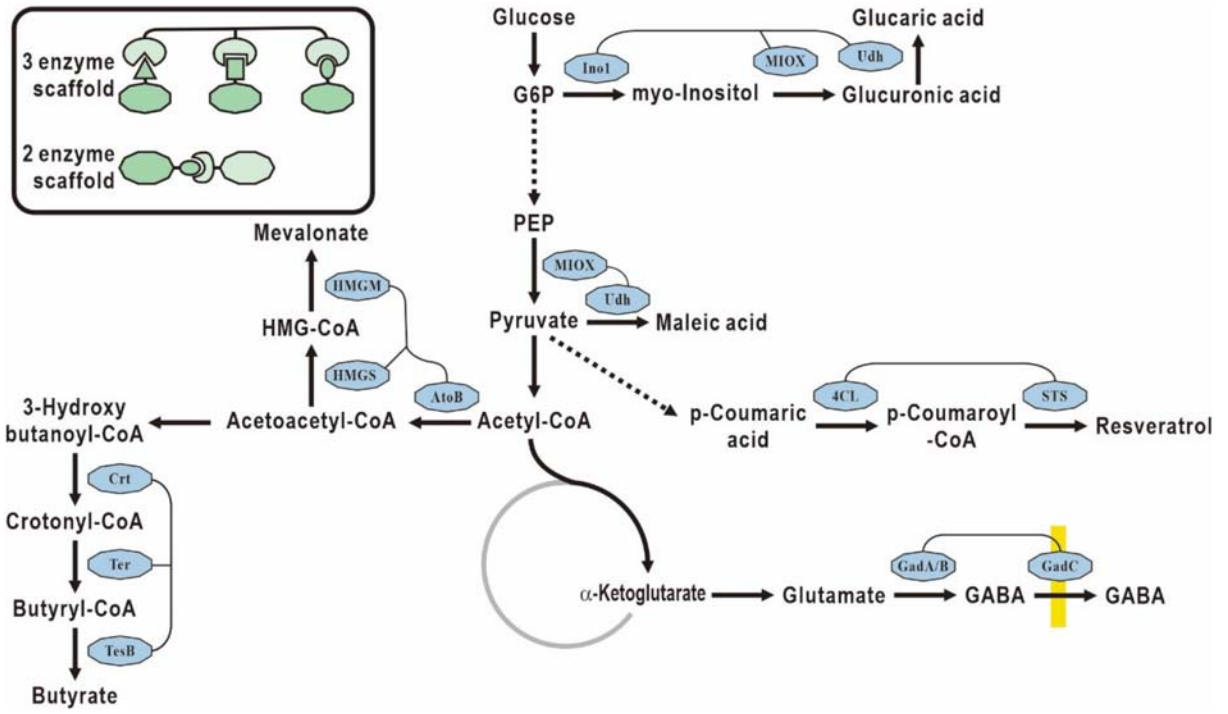

Fig. 2. Application of scaffolding proteins to metabolic engineering**.** Enzymes performing the sequential multistep transformation of a substrate are co-localized into the scaffold. AtoB: acetoacetyl-CoA thiolase, HMGS: hydroxymethyl glutaryl-CoA synthase, HMGR: hydroxymethyl glutaryl-CoA reductase, Ino1: myoinositol-1-phosphate synthase, MIOX: myoinositol oxygenase, Udh: uronate dehydrogenase, Crt: 3-hydroxybutyryl-CoA dehydratase, Ter: trans-enoyl-coenzyme A reductase, TesB: acyl-CoA thioesterase II, 4CL: 4-coumarate: CoA ligase, STS: stilbene synthase, GadA/B: glutamate decarboxylase, GadC: glutamate/GABA antiporter, GABA: gamma-aminobutyric acid, PEP: Phosphoenolpyruvate.

Table 1. Application of peptide motifs and cognate adaptor domains in metabolic engineering

Metabolites	Enzyme pathways	Scaffold model	Host	Titer	Reference
Glucaric acid	Myoinositol-1-phosphate synthase, myoinositol oxygenase, uronate dehydrogenase	SH3/PDZ	*E. coli*	2.37 g/L	[12]
Mevalonate	Acetoacetyl-CoA thiolase, hydroxymethyl glutaryl-CoA synthase, hydroxymethyl glutaryl-CoA reductase	GBD1/SH3/ PDZ2	*E. coli*	5 mM	[17]
GABA	Glutamate decarboxylase, glutamate/GABA antiporter	SH3	*E. coli*	1.01 g/L	[57]
GABA	Glutamate decarboxylase, glutamate/GABA antiporter	SH3/PDZ	*E. coli*	5.26 g/L	[58]
Resveratrol	4-coumarate: CoA ligase, stilbene synthase	SH3/PDZ	*S. cerevisiae*	14.4 mg/L	[14]
Butyrate	Acetoacetyl-CoA thiolase, 3-hydroxy butyryl-CoA dehydrogenase, 3-hydroxy butyryl-CoA dehydratase, trans-enoyl-coenzyme A reductase, acyl-CoA thioesterase II	GBD/SH3/ PDZ	*E. coli*	7.2 g/L	[52]

GABA: gamma-aminobutyric acid, PDZ: postsynaptic density 95/discs large/zona occludens-1, SH3: Src homology 3, *E. coli*: *Escherichia coli, S. cerevisiae: Saccharomyces cerevisiae.*

3.1. Mevalonate

Mevalonate has been widely used in cosmetics as a building block to produce sustainable polymers (Table 1) [46]. Mevalonate is present in eukaryotes, archaea, and some prokaryotes. Since 1956, mevalonate has been produced by fermentation technology [47]. However, the best-identified organism for its production, namely *Saccharomycopsis fibuligera* NRRL Y-7069, exhibits a low mevalonate titer (0.9 g/L) [48].

One of the most prominent examples of peptide motif and cognate adaptor domains in metabolic engineering is the scaffold system engineered for the three-step synthesis of mevalonate [49]. From acetyl-CoA, this system is comprised of three enzymes, namely acetoacetyl-CoA thiolase (AtoB), hydroxymethyl glutaryl-CoA synthase (HMGS), and hydroxymethyl glutaryl-CoA reductase (HMGR), respectively.

There are only the AtoB enzyme is native to the host *E. coli* system, the other two enzymes were imported from *S. cerevisiae*. The scaffold was constructed by flexibly connecting three domains, GBD, SH3, and PDZ with their linkers to AtoB, HMGS, and HMGR. The scaffold advantage helps metabolic avoid flux imbalances and leads to an increase the overall production. By searching for the different architectures of the scaffold, they revealed the best concept for this system to be GBD1–SH32–PDZ2, which indicated that one GBD domain was linked to two SH3 and two PDZ domains. 77-fold mevalonate product yield was significantly increased.

3.2. Glucaric acid

D-glucaric acid known as saccharic acid was classified as a top value-added production chemical from biomass [50]. However, the complex biological route for glucaric acid production requires new strategies involving high selectivity for the product. D-glucaric acid produces from D-glucose, via synthetic scaffold protein, by co-expressing three enzymes myoinositol-1-phosphate synthase (Ino1) from *S. cerevisiae*, myoinositol oxygenase (MIOX) from mouse, and uronate dehydrogenase (Udh) from *Pseudomonas syringae* in *E. coli* [12]. When compared to the original system, the scaffold system which connected the two enzymes Ino1 and MIOX, equipped with the respective peptide ligand sequences, significantly raised the product titer to three-fold (Table 1) [17]. Optimization of the system by varying the number of cognate domains, including Udh, allowed a production increase of about ~50% [12].

3.3. Butyrate

Butyrate, a 4-carbon, short-chain, fatty acid, is a major carbon source for colonic epithelium. Butyrate functions as a signaling molecule in various human metabolic and immune system pathways [51]. It is used in the chemical, food, pharmaceutical, and plastic industries [52]. Industrial production of butyrate by microbial fermentation has been investigated using species of *Clostridium* [52]; however, its prospects are limited by the lack of genetic engineering tools [53].

Engineered *E. coli* with the same scaffolding domains, including GBD, SH3, and PDZ, were used to increase butyrate production. The biosynthetic pathway was completed by five enzymes, AtoB, 3-hydroxybutyryl-CoA dehydrogenase (Hbd), 3-hydroxybutyryl-CoA dehydratase (Crt), trans-enoyl-coenzyme A reductase (Ter), and acyl-CoA thioesterase II (TesB). In the *E. coli* host, a domain scaffold was created and overexpressed. The variation of domain frequency scaffold reported that butyrate production increased three-fold.

3.4. Resveratrol

Resveratrol (3,5,4′-trans hydroxy stilbene), well-known for its presence in red wine, is one of the most widely studied plant-produced polyphenols. In the host strains *S. cerevisiae*, by composing GBD, SH3, and PDZ domains with two enzymes, 4-coumarate: CoA ligase and stilbene synthase which were covalently attached to SH3 and PDZ peptide ligands. The product yield increased five-fold when compared with the non-scaffold proteins system and also 2.7 fold yield increased when compares to the fusion protein approach.

3.5. Gamma-aminobutyric acid

In biotechnology industries, GABA is a non-protein amino acid that is used as a monomer to produce the biopolymer nylon-4 [54,55]. GABA also acts as a neurotransmitter in the central nervous system and exerts several beneficial physiological functions, such as hypotensive induction, diuretic, and tranquilizing effects [56]. Due to its various beneficial effects, particularly blood pressure reduction, anxiety inhibition, and metabolic function acceleration, GABA is used extensively in pharmaceuticals and functional foods. Due to the low production cost of using *E. coli* in industrial fermentation, it is a suitable host for engineering its pathway to produce a high GABA yield [57].

In the *E. coli* BL21 strain, enhanced GABA production was reported upon overexpression of glutamate decarboxylase (GadA/B) enzyme from *Lactobacillus brevis* [55]. The exploitation of domain-ligand interactions is also done beside the creation of multiple adaptor domains contained in a scaffold protein [58]. To enhance GABA production in *E. coli*, the enzyme GadA/GadB was attached to the membrane protein glutamate/GABA antiporter (GadC) via the SH3 domain, and the peptide ligand was separated with flexible linkers. GABA productivity subsequently increased 2.5-fold.

4. Conclusions and Future Perspectives

The synthetic protein scaffold strategy has enormous potential for application in next-generation pathway engineering studies. When pathway enzymes are not closely associated with each other, the metabolic flux towards the target pathway is reduced, resulting in a decrease in the production of target metabolites. The studies reviewed in this manuscript demonstrate that the introduction of a synthetic scaffold complex between pathway enzymes can closely co-localize the enzymes, thereby increasing the metabolic flux to the target pathway and boosting the production of target metabolites. Consequently, we believe that the synthetic

protein scaffold strategy can be applied to chemical networks that use a series of biochemical reactions and enzymes to convert raw materials into target products. Additionally, metabolic engineering strains can be developed to produce novel medicinal products, such as drugs and vaccines.

Acknowledgements

This work was supported by the 2023 Research Fund of the University of Ulsan, Republic of Korea.

Ethical Statements

The authors declare no financial or commercial conflict of interest.
Neither ethical approval nor informed consent was required for this study.

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