A review on chimeric xylanases: methods and conditions

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Abstract Multi-functional enzymes are one of the nature's solutions to facilitate metabolic pathways, thus several reactions are regulated and performed simultaneously on one polypeptide chain. Inspired by nature, artificial chimeric proteins have been designed to reduce the production costs and improve the performance. One of the interesting applications of this method is in the plant-based industries such as feed additive, waste treatment, biofuel production, and pulp and paper bleaching. In fact, the heterogeneous texture of plants needs using a combination of different enzymes to achieve an optimal quality in the manufacturing process. Given that xylans are the most abundant non-cellulosic polysaccharides in nature, xylanases are widely utilized in the mentioned industries. In this regard, several studies have been conducted to develop the relevant chimeric enzymes. Despite the successes that have been attained in this field, misfolding, functional or structural interference, and linker breakage have been reported in some cases. The present paper reviews the research to introduce the prerequisites to design an appropriate chimeric xylanase.

Keywords Domain insertion · Linker · Protein engineering · Tandem fusion

Introduction

Xylanases (EC 3.2.1.8) as hemicellulose-degrading enzymes are widely used in various plant-based industries including pulp and paper bleaching, feed additive, dough processing, juice clarification, textile manufacturing, protoplast preparation, degumming of bast fibers, extraction of coffee, oil, and starch, and production of bioethanol, xylose, xylitol, SCPs (single-cell proteins), solvents, and sugar syrups (Beg et al. 2001; Chen et al. 2015).

Due to the heterogeneous nature of plant cell walls, the presence of diverse enzymes is often necessary to complete degradation of them. Accordingly, many microorganisms mainly possess two approaches to facilitate plant decomposition. The first one includes using complexes such as xylanosomes or cellulosomes in which different enzymatic chains have been attached to a non-catalytic scaffold via a cohesin-dockerin interaction (Bayer et al. 2004). In another strategy some bi- or multi-functional enzymes hold several catalytic activities in a single polypeptide chain. Interestingly, the catalysis can be done in one or several domains, such as what has been reported for xylanase–glucanase complex in *Paenibacillus* sp. E18 and *Ruminococcus flavefaciens* 17, respectively (Flint et al. 1993; Shi et al. 2010). Sequence analysis suggests that the multi-functional enzymes have been appeared to improve the performance of enzymatic reactions during evolution. As a consequence, these enzymes expedite intermediate transfer and regulation mechanisms to increase the efficiency of the metabolic pathway (Elleuche 2015).

Inspired by nature, artificial chimeric proteins have been designed for the multi-billion dollar markets. For example, chimeric drugs had sales of 8.3 billion dollars in 2010 (Schmidt 2013). Another promising usage can be in xylanase-based industries in which a combination of different

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enzymes is requisite to obtain the final product with desirable quality (Khandeparker and Numan 2008; Shi et al. 2010). Although a defined mixture of enzymes is used in these industries, it has been illustrated that the chimeric enzymes can show better performance than mixed enzymes (Fan et al. 2009a, b; Guo et al. 2013). Moreover, fused enzymes are helpful in the crowded environment with high viscosity. If the chimeric enzymes catalyze consecutive reaction, the intermediates transfer straight to the next active site in the corresponding enzyme. Therefore, the intermediates are consumed before distribution in the solution, and the reaction is completed with a higher efficiency in a lesser time. Above all, the production of fusion enzymes is cheaper and easier (Elleuche 2015). In addition, the enzymatic characteristics such as stability can be improved in fusion enzymes (Diogo et al. 2015). Despite the mentioned advantages; lack of knowledge can cause problems such as misfolding, functional or structural interference and/or breaking protein–protein connectors. Consequently, this article reviews the requirements to design a suitable chimera.

Considerations to create a chimeric protein are primarily affected by the type of fusion approach. Proteins can be fused by three methods: tandem fusion, domain insertion and post-translational conjugation. The latest one mostly connects proteins using the bifunctional reagents (e.g., glutaraldehyde) which are able to make a covalent binding with the reactive functional groups on proteins (e.g., –SH, –NH₂, and –COOH) (Yu et al. 2015). This has been used fewer than other methods and there is almost no report to build the chimeric xylanases by this system. Therefore, we focus on the first two methods in the following.

Chimeric xylanases with domain insertion

Domain insertion exists in about 9% of natural multi-domain proteins, especially in α/β classes (Yu et al. 2015). The most critical step in this method is selecting an appropriate cut-site to avoid disruption of the protein conformation. Hence, a domain has to be inserted in the places that are not directly involved in enzyme activity, such as surface loops and turns. However, domains should not interfere with each other to find the correct folding after insertion. Generally, computational methods such as SCHEMA algorithm can be used to take a closer investigation into the impact of a segment entrance on the protein conformation (Smith and Arnold 2014). Apart from the mentioned challenges, this method has a notable advantage as a result of using one domain instead of whole enzyme. In this way, a severe increase in the molecular weight, observed in tandem fusion, is not expected. This not only reduces metabolic burden on the host cells, but also is more

appropriate for the enzyme penetration through the polymeric substrates like hemi-cellulose. On the other hand a lower activity is predicted because of the steric hindrance resulting from getting too close. Thus, it has been suggested using a domain with its surrounding areas for insertion.

One example of this kind of chimeric xylanases was produced by insertion of the whole sequence of xylanase (XynA) into a surface loop of thermophile laccase (CotA). Both enzymes were from *Bacillus subtilis*. The chimeric enzyme showed nearly two times greater laccase activity. In addition, the solubility and the resistance to proteolytic attacks were enhanced. Although the optimum pH and temperature of the chimeric enzyme was almost as the same as the parent enzymes, the xylanase thermostability had significantly improved. In fact, the N- and C-terminal regions of CotA had a considerable influence on the thermostability of the surrounded XynA (Ribeiro et al. 2011).

In addition to catalytic domains, other regions can also be transferred for domain study and/or physicochemical advancement. Modules of the family F/10 xylanases have drawn the most attention in this regard. A module embraces a compact cluster of 10–40 adjacent amino acid residues which are supposed to act as independent functional blocks (Ahsan et al. 2001). Endo- β -1,4-xylanase from *Cellulomonas fimi* (Cex) is one of the well-characterized xylanases that contains 22 modules on the basis of the crystal structure analysis. It has been demonstrated that the modules 4, 6, 7, 10, 15, 19 and 20 of Cex are responsible for substrate binding. On account of this, several articles have reported DNA shuffling between Cex and its homologues xylanases. For example, an exchange of M10 between Cex and FXYN (from *Streptomyces olivaceoviridis* E-86) boosted the catalytic activity of FXYN. Since the M10 is conserved among family 10 xylanases, its replacement leaves no adverse effects on the folding (Kaneko et al. 1999; Mesta et al. 2001). In another study, a tenfold increase in the catalytic activity was observed by substitution of M15–M22 in FXYN with the respective modules from Cex. Interestingly, an inactive enzyme was obtained by shuffling of M1–M14 (Kaneko et al. 2004). Besides the catalytic activity, a thermostable Cex was created by replacing the modules 4 and 5 with the corresponding modules of thermophile XylA from *Thermomonospora alba* (Wang and Xia 2007). The same result was achieved using self-priming overlapping PCR for the flexible modules 3, 4, 14 and 15 XylA and FXYN. In spite of the improvement in temperature and pH profiles of both FXYN-M3/4-XylA and FXYN-M14/15-XylA, the thermal stability only was noted for FXYN-M3/4-XylA. It suggests that the modules 4–14 of XylA may be responsible for inducing thermal stability (Ahsan et al. 2001).

A survey on the relationship between alkaline xylanase regions and pH-activity was done by overlapping PCR between XynA and XynB from *Bacillus halodurans* C-125 and *Clostridium stercoararium* F9, respectively. Both xylanases have a homology in four regions: N-terminal region (A), proton donor region (P), catalytic nucleophile region (N), and C-terminal region (C). Among these regions, A and P sections indicated a significant importance to activity in alkaline pH (Nishtmoto et al. 2002).

A combination of alkaline and thermostable xylanases for pre-bleaching of paper pulp was generated by DNA shuffling between G53 and G41, two mutants of xynA from *Thermomyces lanuginosus* xylanase DSM 5826. G41 was a thermostable xylanase with 75% activity at 80 °C, and 40% activity at pH 10. On the contrary, G53 was an alkaline stable xylanase with 93% activity at pH 10, and 10% activity at 80 °C. The result of DNA shuffling, which inherited mutations from both parents, showed 85% stability at 80 °C and 60% stability at pH 10. Interestingly, the authors noted that the thermostable variants displayed higher arginine content and lower activity. It has been suggested that arginine can affect the stability of protein by making salt bridges on the protein surface (Stephens et al. 2014).

Although most of the researches were done on the family 10 xylanases, here is a report on the relevant regions to alkaline activity of BadX from family GH11. For this purpose a gene shuffling for five segments was performed between BadX from *Bacillus agaradhaerens* and XynB6 from *Dictyoglomus thermophilum* by degenerate oligonucleotide gene shuffling (DOGS) method. However, the results indicated no significant improvement in alkaline pH characteristics of the enzymes, thus the authors concluded that a few mutations is not adequate to alter the pH optimum of family 11 xylanases (Gibbs et al. 2010). Conversely, a thermostable xylanase was created in GH11 family by substituting the N-terminus of SoxB from *Streptomyces olivaceovirdis* with TfxA from *Thermomonospora fusca*. The half-life of the produced xylanase was about 493 min in 70 °C which was nearly 136-fold higher than wild SoxB (Zhang et al. 2010).

End to end fusion; simple and prevalent method

Tandem fusion is the most common method to construct chimeric proteins because it is less dependent on the knowledge of protein structure and using special equipment. In this approach two or more sequences are connected through end-to-end fusion with or without a linker. For this purpose, the first gene should be free of stop codon and the last gene should be without ribosome binding site and signal peptide sequence. Although a reduction in

expression level is probable as a result of sequence length increasing, this restriction can be controlled by codon optimization, adjustment of the mRNA secondary structure, and putting a well-expressed protein at the N-terminal domain (Elleuche 2015). Finally, it is suggested to check the structure of the chimera with different software, such as I-TASSER, MOLMOL and PSIPRED.

The chimeric xylanases produced by end-to-end method along with the alternations which have taken place in their properties are summarized in Table 1. As it is clear, most of the products showed no significant variation in the optimum temperature and pH. However, presence of more alkaline enzyme at N-terminal region may decrease the optimum pH of chimeric enzyme.

It should be noted that the chimeras in Table 1 have been created from two enzymes, while some examples exist with more enzymes. For instance, XAR-XYN embraces xylosidase-arabinosidase (XAR) and xylanase (XYN) activities (Wang et al. 2010; Xue et al. 2009). In addition to enzyme fusion, there is a report for xylanase-ELPs (Elastin-like polypeptides) coupling. This connection increased the optimum temperature, specific activity, and storage capability of enzyme (Li and Zhang 2014). In another example, an improvement in the optimum temperature and specific activity along with a decline in thermostability was created by linking OsmC-region (osmotically induced protein) to Xyn8 of *Pseudoalteromonas arcica* (Elleuche et al. 2011).

Although tandem fusion seems simple, several parameters have to be considered before getting started; such as sequence arrangement and style of connection. The position of enzymes is affected by the strength of the translation initiation, flexibility of the connecting domains, and conformation, structure and physicochemical property of each parent protein. According to the N-end rule, the N-terminal residues have a determining role in the half-life of a protein. For this reason, putting the more thermostable enzyme in the N-terminus of chimera is desirable to improve the thermal stability of the next enzyme (Zhang et al. 2010). Moreover, it has been illustrated that the N-terminal domain of XynA needs being free for making a significant effect on the thermostability of the adjacent enzyme (Diogo et al. 2015).

One of the critical points in making chimeric enzymes refers to protein linkage. The easiest way is end-to-end fusion in which the unstructured C- or N-terminal of the enzymes acts as a bridge and provides enough space between catalytic domains. However, a non-functional protein can be made if the terminals are short, rigid or essential for protein activity. These have contributed to steric hindrance, misfolding, reduction in protein dynamics, and prevention of interaction between protein subunits. In this situation an external sequence called linker can be

Table 1 Chimeric xylanases constructed by tandem fusion along with the physicochemical properties

Character of N-terminal enzyme					Linker	Character of C-terminal enzyme					References		
k_{cat}	K_m	Activity	Tp (°C)	pH	N-terminal Enzyme		C-Terminal enzyme	pH	Tp (°C)	Activity	K_m	k_{cat}	References
			50▼	6.5	Endo-1,4- β -xylanase	ETITGEVDDC EYEQAQQQAG PEVTYE	β -xylosidase	6.5	35▲				(Diogo et al. 2015)
			65	8▼	β -D-xylanase	GGGGADQLAIGPMYNQVVYQYPN	β -D-xylosidase	6	60				(Fan et al. 2009a)
			65	8▼	β -D-xylanase	GGGGADQLAIGPMYNQVVYQYPN	α -L-arabinofuranosidase	6	70				(Fan et al. 2009a, b)
	0.75▲	0.72▼	60	5	Feruloyl esterase A	GSTYSSGSSSGSGSSSS	XYNB	5.5▲	50▼	386▲	6.6▲		(Levasseur et al. 2005)
173.7▼	7.87▼	3.73▼	50	6.5	XynA	4-glycine linker	bglS (lichenase)	6.5	50	3.65▲	3.39▼	197.2▲	(Cota et al. 2013)
	7▲	21,122.7▼			Xylanase (Xyl11)	(GGGS) ₄	Endo-1,4- β -mannanase			1231.6▼	18.1		(Guo et al. 2013)
		136.3▲	62▼	6▼	XynCDBFV	(GGGS) ₂	Acetylxyylan esterase						(Pai et al. 2012)
1941.5▲	15.3▲	727.21▼	70	7▼	Xyl10g	(GGGS) ₂	Endoglucanase (Cel5B)	4	50▲	482.94▼	31.1▼	11,439.83▼	(Kim et al. 2015)
		642	50	7	β -1,4-endoglucanase (Endo5A)	(GGGS) ₂	Xyl11ID	7	50	389▲			(Adlakha et al. 2011)
6565▲	4.2▼		40	9	β -glucanase	Without linker	Xylanase	9	60▼		4.95▲	6491▼	(Lu and Feng 2008; Lu et al. 2006)
49.2▲	12.3▼		47▲	3.8	Xylanase	PEVLPLPK ESRISEGEAV VVG	Glucanase	3.8	96		1.87▲	2.6▲	(Liu et al. 2012)
949▼	2.36▲	324▼	50	3.4	XynA	AAAAAA	β -1,3-1,4-glucanase (Bs-Glu)	6.4	40	1181.8▼	4.06▲	11,149▼	(Qiao and Cao 2012)
	7.41	75▲	80▼	7▼	XylIT	VDKTKYTAS	Cel5A	6	90▼	1431▲	7.41		(Rizk et al. 2015)

No data have been reported for the blank cells

Symbols ▲ and ▼, respectively, represent increase and decrease in the parameters after constructing chimeric enzyme. Those are asymptomatic were without change

used. A database of various linkers has been presented by the Centre for Integrative Bioinformatics VU (IBIVU) at the Vrije University of Amsterdam (<http://www.ibi.vu.nl/programs/linkerdbwww/>). In addition to online databases, a genetic algorithm can be used to determine the appropriate connector (Zhang et al. 2009). Generally, linkers can be categorized based on the length, amino acid content, hydrophobicity, secondary structure, solvent accessibility and sensitivity to proteases. However, the following classification is more prevalent and comprehensive form (Yu et al. 2015).

1. **Flexible linker** Small amino acids such as Gly and Ala can make the flexible linkers. The presence of polar amino acids like Ser, Thr, Glu and Lys lead to stability of the linker in the aqueous solvents by making hydrogen bond (Chen et al. 2013). The most popular flexible linker is (GGGGS)_n (usually $n \leq 6$) which forms a loop. However, this glycine-rich region can reduce the level of protein expression by creating a stable mRNA secondary structure. Furthermore, the GC-rich content (codon GGN for glycine) may be unacceptable for some microorganisms (Lu and Feng 2008). It is worth to note that the humdrum rhythm of Gly in (GGGGS)₂ reduces the functional activity of enzymes. Thus, using a combination of other small amino acids such as Ala and Ser can help to overcome this problem (Wang et al. 2010).
2. **Rigid linker** This is useful when a defined distance is necessary between enzymes. As a result of being tough and stable, they can inhibit interactions between proteins and support correct folding. The most common type is (EAAAK)_n (usually $n \leq 6$), which forms helices and salt bridges between its Glu⁻–Lys⁺. Another type is proline-rich sequences (XP)_n, where X can be any amino acid, preferably Ala, Lys or Glu. This kind of linkers naturally has been discovered in proteases and pyruvate dehydrogenases. Since Pro has a circular and solid structure, it makes a firm and independent connection. Moreover, lack of hydrogen on the amide group of Pro prevents from interaction with other amino acids (Chen et al. 2013).
3. **Cleavable linker** These linkers apply to separation of chimeric proteins when exposed to solvents or proteases. MEROPS is a database to determine the sensitivity to peptidase.

In contrast to the flexible and rigid linkers, there is no report for the usage of the cleavable linkers in the chimeric xylanases (Table 1). Therefore, the first two categories will be discussed in the following.

A comparison between [GGGGS]_n and [EAAAK]_n linkers ($n = 2-4$) has illustrated that xylanase-mannase chimeras which were created by [EAAAK]₃ had a better

thermostability. However, the flexible linker with $n = 3$ causes more catalytic activity. In addition, K_m was greater with rigid linkers (Guo et al. 2013). Similar results were obtained by the mentioned linkers ($n = 1-3$) for xylanase-gluconase chimeras (Lu and Feng 2008). Amino acid composition of linkers can also affect post-translational modifications and thus protein stability. A good example of this was reported by Levasseur et al. (2005) in which the proteolytic cleavage of linker was avoided using a hyperglycosylated linker. Furthermore, the authors believed that the glycosylation of linker can be useful for increasing the secretion and production yield of recombinant proteins (Levasseur et al. 2005).

In addition to amino acid content, length of linkers has been investigated. Researches have shown that the solvent accessibility and hydrophilicity are related to the length of linker. Accordingly, access to solvent for long (average length of 21.0 ± 7.6 residues), medium (9.1 ± 2.4) and short (4.5 ± 0.7) linkers are about 42.5, 26.3 and 14.4%, respectively (Chen et al. 2013; George and Heringa 2003). Although a linker should be long enough so that each enzyme acts independently, the possibility of breakage increases if the linker be too long (Qiao and Cao 2012). It has been demonstrated that a 31 amino acids linker is suitable not only to prevent collision of two enzymes, but also to make great flexibility to provide free access and binding to the substrate (Diogo et al. 2015). In another study, Fan and coworkers found that a five glycine residues linker cannot make enough distance between two enzymes; therefore, they used a 24 residues linker (Fan et al. 2009a). Although the mentioned results provide useful information for designing an appropriate linker, further investigation may be required depending on the aim of study and properties of the target protein.

Conclusion

The simple structure and diverse industrial usages of xylanases have led to perform several studies to create relevant chimeric enzymes to reduce the production costs and improve the characteristics of enzymes. The two main methods discussed here were domain insertion and tandem fusion. In both methods, maintenance of the protein conformation and inhibition of overall interference are the most critical criteria. In this regard, selection of cut-site is the important step in domain insertion and mostly takes place in loops or turns that does not have a direct effect on enzyme activity or folding. However, in domain replacement a high similarity and accordance between enzymes is necessary. Most of the reports in this area have been done on the family 10 xylanases because modules of Cex from this family have been identified and can be used to advance

other xylanases from the same family without making misfolding (Kaneko et al. 1999). However, this method requires knowledge about the structure of enzymes, and also is associated with high possibility of steric hindrance, folding interference, and inactivity, thus most of the researchers employ tandem fusion. The features of parent enzymes as well as the communication bridge have significant effects on the results. When a suitable terminal domain does not exist for direct connection, a linker is used. Linkers have various applications in biotechnology, such as the production of bifunctional enzymes and antibodies and as a means of FRET analysis (Chichili et al. 2013). These linkers often are derived from nature. Amino acid residues that are common to all natural linker are Pro, Thr, and Gln (Chen et al. 2013). Generally, a linker should be without a protease cleavage site and enough distances provided between the two independent activities of each enzyme fusion. According to the studies, an average length of 15 amino acid residues is suitable for connection. Selection of the type of linker also depends on the purpose of the research. It is proposed here to use rigid and flexible linkers for thermal stability and enhanced activity, respectively. Although there is no report for cleavable linkers in the creation of chimeric xylanases, they can at least be used to reduce the cost of production and purification of enzymes.

In spite of the selected method, terminal domains, N-terminal domains are especially important in protein stability. Therefore, it is suggested that the more stable enzyme is put in this position. In addition, using post-translational modification sites such as N-acetylation or glycosylation sites can improve the enzyme stability.

Compliance with ethical standards

Conflict of interest The author declares that there is no conflict of interest regarding the publication of this paper.

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