Poly-γ-glutamic acid: production, properties and applications

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Poly-γ-glutamic acid (γ-PGA) is a naturally occurring biopolymer made up of repeating units of L-glutamic acid, D-glutamic acid or both. γ-PGA can exhibit different properties (conformational states, enantiomeric properties and molecular mass). Owing to its biodegradable, non-toxic and non-immunogenic properties, it has been used successfully in the food, medical and wastewater industries. Amongst other novel applications, it has the potential to be used for protein crystallization, as a soft tissue adhesive and a non-viral vector for safe gene delivery. This review focuses on the production, properties and applications of γ-PGA. Each application of γ-PGA utilizes specific properties attributed to various forms of γ-PGA. As a result of its growing applications, more strains of bacteria need to be investigated for γ-PGA production to obtain high yields of γ-PGA with different properties. Many medical applications (especially drug delivery) have exploited x-PGA. As γ-PGA is essentially different from x-PGA (i.e. it does not involve a chemical modification step and is not susceptible to proteases), it could be better utilized for such medical applications. Optimization of γ-PGA with respect to cost of production, molecular mass and conformational/enantiomeric properties is a major step in making its application practical. Analyses of γ-PGA production and knowledge of the enzymes and genes involved in γ-PGA production will not only help increase productivity whilst reducing the cost of production, but also help to understand the mechanism by which γ-PGA is effective in numerous applications.

Introduction

Polyglutamic acid (PGA) is a biodegradable, non-immunogenic and unusual anionic homopolyamide that is made of D- and L-glutamic acid units (Shih & Van, 2001) (Fig. 1).

PGA can be differentiated into two isoforms, α-PGA and γ-PGA, depending on the attachment of the carboxy group (α and γ, respectively). α-PGA is synthesized chemically by nucleophile-initiated polymerization of the γ-protected N-carboxyanhydride of L-glutamic acid. Microbial production of α-PGA is difficult and the polymer can only be produced by recombinant technology (Buescher & Margaritis, 2007). γ-PGA has been produced extensively using bacteria, especially those of Bacillus species. It can either be composed of only L-glutamic acid residues (γ-L-PGA), only D-glutamic acid residues (γ-D-PGA) or both L- and D-glutamic acid residues (γ-LD-PGA). γ-PGA is different from other proteins, because glutamate is polymerized inside the cell via the γ-amide linkages and thus is synthesized in a ribosome-independent manner (Akagi et al., 2007; Bodnár et al., 2008). Hence, substances that inhibit translation of proteins, such as chloramphenicol, have no effect on the production of γ-PGA. Due to the γ-linkage of its component glutamate residues, γ-PGA is resistant to proteases, which cleave α-amino linkages (Candela & Fouet, 2006). γ-PGA can exist either in the water-insoluble free acid form or as its salt with a variety of cations (Na+, Mg2+, K+, NH4+ or Ca2+), which is completely soluble.

γ-PGA was first discovered by Ivonics and Bruckner (1937a, b) when a capsule of Bacillus anthracis was released into the medium upon autoclaving (Shih & Van, 2001). Another naturally occurring source of γ-PGA is the mucilage of natto (fermented soybeans – a traditional food in Japan), which contains a mixture of γ-PGA and fructan produced by Bacillus subtilis Sawamura (Shih & Van, 2001; Candela & Fouet, 2006). γ-PGA is produced mostly by Gram-positive bacteria, which include the genus Bacillus. It has also been reported that at least one Gram-negative bacterium (Fusobacterium nucleatum), some archaea and eukaryotes have the ability to produce γ-PGA (Candela et al., 2009; Weber, 1990; Hezayen et al., 2001). γ-PGA has also been found in neurons of mice where it was covalently

Abbreviations: GGT, γ-glutamyl-transpeptidase; GPC, gel permeation chromatography; NK, natural killer; NP, nanoparticle; PEC, polyelectrolyte complex; PGA, polyglutamic acid.
linked to tubulin (Eddé et al., 1990). Efforts have been made to insert the genes responsible for γ-PGA production into Escherichia coli and plants such as tobacco to gain more knowledge regarding the molecular mechanism of γ-PGA production (Ashiuchi et al., 1999a; Tarui et al., 2005), and pgsB, pgsC and pgsA genes were identified as essential for its production. Recently, Cao et al. (2013) also cloned and co-expressed the pgsBCA genes and glutamate racemase gene (racE/glr) into E. coli, and reported that the engineered E. coli strains had the ability to synthesize γ-PGA with excellent ω-glutamate content due to racE integration. Here, we provide a brief updated review on the production, properties and applications of bacterially produced γ-PGA, and give a brief summary of the mechanism of synthesis of γ-PGA.

**Function of γ-PGA**

Why do organisms produce γ-PGA? The function of γ-PGA depends on the organism producing it and the environment the organism inhabits. The function also depends on whether the γ-PGA is peptidoglycan bound or released. If the γ-PGA is peptidoglycan bound, it may help confer virulence or it can act as a source of glutamate under conditions of starvation (Kimura et al., 2004; Kocianova et al., 2005). If it is released into the environment, γ-PGA can help the organism survive under adverse conditions (McLean et al., 1990).

It has been shown that the capsules of virulent strains of B. anthracis contain solely γ-D-PGA (Tomcsik & Szongott, 1933 in Candela & Fouet, 2006). The D enantiomer in B. anthracis capsule makes the bacterium non-immunogenic (Zwartouw & Smith, 1956). The γ-PGA protects the bacterial cells against phage infections and also prevents antibodies from gaining access to the bacterium (Mesnage et al., 1998). Likewise, Staphylococcus epidermidis also synthesizes surface-associated γ-PGA (Kocianova et al., 2005), which protects it against antimicrobial peptides. In both cases, γ-PGA helps the pathogenic bacteria to escape phagocytosis, hence contributing to virulence. In some cases, γ-PGA also acts as a source of glutamate when bacteria are starved in the late stationary phase (Kimura et al., 2004).

Unlike B. anthracis, certain soil bacteria release γ-PGA into the environment for the sequestration of toxic metal ions, increasing their resistance to adverse environments (McLean et al., 1990). Planococcus halophilus, Sporosarcina halophila and Natrialba aegyptiaca specifically use γ-PGA to decrease high local salt concentrations, which helps them to survive in hostile environments (Kandler et al., 1983; Hezayen et al., 2001). Eukaryotic organisms such as Cnidaria are also known to produce γ-PGA. These marine animals have nematocysts (stinging cells) that use them explosively for prey capture, locomotion and protection. Large amounts of γ-PGA help to trigger this explosive reaction (Weber, 1990). As mentioned earlier, the presence of γ-PGA has also been shown in neurons of mice (Eddé et al., 1990) where it is thought to play a role in the regulation of microtubule dynamics by modifying the interaction of tubulin with tubulin-associated proteins and Ca2+. (Eddé et al., 1990). Bacillus amyloliquefaciens C06 uses γ-PGA to improve both its ability to form biofilm and motility, by causing cells to stick together in a coordinated pattern and absorbing essential nutrients needed for motility from the environment, respectively (Liu et al., 2010).

**Mechanism of synthesis of γ-PGA**

One of the major challenges of making γ-PGA usage applicable in industry is its cost. According to Sung et al. (2005b), γ-PGA is several tens to hundreds fold more expensive than the conventional materials it is envisioned to replace. Reducing the cost of production is the only foreseeable solution to this problem. Designing mass production systems for γ-PGA would be a major step towards a potent solution. To achieve this, one needs to obtain thorough knowledge of how different factors affect the yield of production. Information about genes and enzymes involved in γ-PGA production would certainly help in manipulating organisms for more efficient production of γ-PGA.

The past 15 years have seen a rise in research in this direction (Ashiuchi & Misono, 2002; Sung et al., 2005b; Candela & Fouet, 2006; Buescher & Margaritis, 2007) and genes that play a role in every step of γ-PGA production have been identified. This section attempts to provide an understanding of the current knowledge of the mechanism of γ-PGA synthesis.

A biosynthetic pathway for the production of γ-PGA has been proposed (Fig. 2). L-Glutamic acid units that make up γ-PGA can be derived from two sources. They can be obtained via the glutamic acid biosynthetic pathway either exogenously or endogenously. Endogenous production of L-glutamic acid requires conversion of a carbon source via acetyl-CoA and TCA cycle intermediates. α-Ketoglutaric acid from the TCA cycle serves as a direct precursor of glutamic acid synthesis (Ko & Gross, 1998; Rehm, 2009). Exogenous L-glutamic acid can be converted to L-glutamine with the help of the enzyme glutamine synthase. L-Glutamine is a precursor of γ-PGA as well. The process of γ-PGA synthesis can be seen to have four distinct stages: γ-PGA racemization, γ-PGA polymerization, γ-PGA regulation and γ-PGA degradation.
As mentioned earlier, \(\gamma\)-PGA can have L, D or both L and D enantiomers of glutamic acid in varying amounts. To incorporate D-glutamic acid into the growing chain of \(\gamma\)-PGA, it needs to be obtained from L-glutamic acid (exogenously supplied or produced \textit{de novo}) by a racemization reaction.

\textit{B. subtilis} has two homologues of the glutamate racemase gene, \textit{racE/glr} and \textit{yrpC} (Ashiuchi \textit{et al.}, 1998, 1999b). The functions of these genes are still uncertain as there are differing reports on the importance of each gene (Ashiuchi \textit{et al.}, 2003b; Kada \textit{et al.}, 2004; Kimura \textit{et al.}, 2004). \textit{RacE} is a cytosolic enzyme with a high selectivity for glutamic acid and a preference for L-glutamic acid (Thorne \textit{et al.}, 1955; Buescher & Margaritis, 2007). According to Kimura \textit{et al.} (2004), \textit{racE} was only important for growth in complex medium, whereas \textit{yrpC} was found to be active when cells were grown on minimal medium. Neither of the two glutamate racemase genes was found to be responsible for the synthesis of \(\gamma\)-PGA even though both seemed essential for D-glutamate catabolism (Kimura \textit{et al.}, 2004).

On the contrary, Kada \textit{et al.} (2004) found \textit{grl} to be essential for converting L-glutamate into D-glutamate for the synthesis of \(\gamma\)-PGA and peptidoglycan as well as growth in \textit{B. subtilis}. Research has, however, shown that Mn\(^{2+}\) affects the enantiomeric composition of \(\gamma\)-PGA (Cromwick & Gross, 1995a, b; Pérez-Camero \textit{et al.}, 1999; Wu \textit{et al.}, 2006). Ashiuchi \textit{et al.} (2004) showed that Mn\(^{2+}\) affected the enantiomeric composition by altering the expression of the \textit{grl} gene. The ratio between L- and D-glutamate in \textit{Bacillus licheniformis} \(\gamma\)-PGA also depends on the concentration of Co\(^{2+}\) and Zn\(^{2+}\). Other researchers have also confirmed the dependence of the enantiomeric composition of \(\gamma\)-PGA on Mn\(^{2+}\) in both \textit{B. licheniformis} and \textit{B. subtilis} (Cromwick & Gross, 1995a; Wu \textit{et al.}, 2006).

\(\gamma\)-PGA polymerization

In the case of \textit{B. anthracis}, the genes involved in \(\gamma\)-PGA synthesis lie on a large plasmid, as opposed to other \textit{Bacillus} species where the genes are present on the chromosome (Ashiuchi \textit{et al.}, 2001a; Shih & Van, 2001).

When \(\gamma\)-PGA is surface associated (as in the case of \textit{B. anthracis}), \textit{cap} (capsule) genes are required for its production. However, for \(\gamma\)-PGA that is released, the \textit{pgs} (polyglutamate synthase) genes come into action (Candela & Fouet, 2006). Both the \textit{cap} and \textit{pgs} gene sets have at least four genes: \textit{capB}, \textit{C}, \textit{A} and \textit{E} or \textit{pgsB}, \textit{C}, \textit{A} and \textit{E} (Fig. 3). The \textit{pgsBCA} genes of \textit{B. subtilis} IFO3336 (\textit{B. natto}) are homologous to the \textit{capBCA} genes of \textit{B. anthracis} (Makino \textit{et al.}, 1989; Shih & Van, 2001). \textit{pgsBCA} has been identified as the sole machinery responsible for \(\gamma\)-PGA synthesis in \textit{Bacillus} species. To prove this, Sung \textit{et al.} (2005b)

\begin{figure}
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\includegraphics[width=\textwidth]{fig2}
\caption{A proposed pathway for the synthesis of \(\gamma\)-PGA. \(\alpha\)-KG, \(\alpha\)-ketoglutaric acid; PGS, polyglutamate synthase. Adapted from Ho \textit{et al.} (2006), Buescher & Margaritis (2007) and Shih & Wu (2009).}
\end{figure}
CapE (a 47 aa peptide) is also responsible and essential for B. subtilis Microbiology disrupted pgsBCA genes in B. subtilis (chungkookjang) creating pgsBCA-null mutants incapable of γ-PGA production. In contrast, Urushibata et al. (2002a) are of the opinion that only pgsB and pgsC are required for the production of γ-PGA. The role of pgsE in the production of γ-PGA is dispensable since pgsB, pgsC and pgsAA at high concentrations were able to form a complex capable of producing γ-PGA even in the absence of pgsE (Candela et al., 2005). On the contrary, Yamashiro et al. (2011) found pgsE to be essential in γ-PGA production as its introduction into the production medium in the presence of Zn$^{2+}$ tripled the production rate of γ-PGA from B. subtilis (chungkookjang). Candela et al. (2005) showed that CapE (a 47 aa peptide) is also responsible and essential for the production of γ-PGA as it appeared to interact with CapA. The unique membrane-bound PgsBCA complex is highly unstable and hydrophobic, which makes isolation of this complex challenging.

The mechanism of polymerization has been shown to be dependent on ATP. The phosphoryl group of ATP is first transferred to a terminal carboxyl group of elongated γ-PGA through substrate-dependent ATP hydrolysis (Sung et al., 2005b). Then, due to a nucleophilic attack of an amino group of glutamic acid on the phosphorylated carboxyl group, an amide linkage is formed. This reaction continues to polymerize γ-PGA at the active site of the synthase complex (PgsBCA). PgsB and PgsC together form most parts of the complex’s catalytic site, whereas PgsA seems to remove the elongated chain from the active site so that the next monomer can be added and may also be involved in transporting γ-PGA (Ashiuchi et al., 2001b; Urushibata et al., 2002a). Activity of PgsBCA was found to be dependent on Mg$^{2+}$ (Ashiuchi et al., 2004). Transportation of γ-PGA outside the cell could be facilitated by a less compact cell membrane with shorter phospholipids (Buescher & Margaritis, 2007).

**γ-PGA regulation**

γ-PGA synthesis in B. subtilis (natto) is thought to be regulated by the ComP–ComA signal transduction system (Tran et al., 2000). Stanley & Lazazzera (2005) further identified a two-part system DegS–DegU, DegQ and SwrA as unusual regulators of γ-PGA production in addition to ComPA. The regulatory effects of DegSU, DegQ and ComPA seem to be transcriptional in response to quorum sensing, osmolarity and phase variation signals, whilst that of SwrA seems to be post-transcriptional (Stanley & Lazazzera, 2005). Osera et al. (2009) examined the relationship between SwrA and DegU, and discovered that the presence of both SwrA and phosphorylated DegU (DegU-P) is required to fully activate the pgs operon and in turn γ-PGA production as the effect of either gene on both pgs transcription and γ-PGA production is negligible. In contrast, Ohsawa et al. (2009) showed that a high level of DegU-P can directly activate pgs expression in place of swrA and high levels of degQ, but still found swrA necessary for γ-PGA production under certain experimental conditions.

To identify the role of DegQ in γ-PGA synthesis, the degQ gene was knocked out in B. subtilis (natto), and suppressor mutants capable of producing γ-PGA synthesis in the absence of degQ were isolated and used (Do et al., 2011). This was compared with domestic strain B. subtilis 168, which is unable to produce γ-PGA due to low transcription of the biosynthetic pgs operon even though it has a higher genetic competency than the wild strain B. subtilis (natto) (Stanley & Lazazzera, 2005; Osera et al., 2009). Do et al. (2011) found that an alteration in degQ drastically prevented the synthesis of γ-PGA in B. subtilis (natto) and downregulated the production of degradation enzymes, which is in line with the report of Stanley & Lazazzera (2005). The degQ gene of B. subtilis (natto) is therefore important for the synthesis of γ-PGA (Do et al., 2011).

**γ-PGA degradation**

A number of enzymes have been shown to be associated with the degradation of γ-PGA. γ-Glutamyl-transpeptidases (GGT) are enzymes that breakdown the transfer of a γ-glutamyl group from a donor species to acceptor (peptides and amino acids) species by developing an intermediate γ-glutamyl enzyme in a transpeptidation reaction (Morelli et al., 2014). The enzyme has the ability to perform exo-hydrolase activity towards γ-PGA, and the released glutamic acid is used by the bacterium as a source of carbon and nitrogen (Kimura et al., 2004; Morelli et al., 2014).
CapD is a part of the GGT family and is required for the covalent anchoring of γ-PGA to the peptidoglycan as well as acting as a depolymerase (Candela & Fouet, 2005). CapD cleaves the γ-PGA and transfers it to either an acceptor molecule or H₂O, resulting in transpeptidation or hydrolysis, respectively (Candela et al., 2014). As γ-PGA can be either anchored to the bacterial surface or released, whilst CapD catalyses the anchorage of γ-PGA to peptidoglycan, PgsS catalyses the release of γ-PGA (Candela & Fouet, 2006). The strains that have pgsBCA, but do not produce γ-PGA, do so because the genes are not translated and not because an inactive gene product is produced (Urushibata et al., 2002b).

Yao et al. (2009) investigated the presence and activity of γ-PGA depolymerase enzyme in B. subtilis NX-2, which is responsible for the depolymerization of γ-PGA in batch culture. The enzyme was seen to be active extracellularly in the culture and was shown to be an endo-hydrolase. The gene encoding the enzyme was ywtD (pgsS). The YwtD protein was obtained in purified form after the gene was cloned and expressed in E. coli. The enzyme was active over a temperature range of 30–40 °C and a pH range of 5–8. At the optimal pH and temperature (5 and 30 °C respectively), a reduction in γ-PGA molecular mass was observed from 1000 to 20 kDa and dispersity decreased as a function of depolymerization time. The enzyme was also seen to be active extracellularly during the late stationary phase. The study demonstrated a mild method for controlled reduction of molecular mass, and could be used as a better alternative to physical and chemical methods of degradation.

Conformation and enantiomeric composition of γ-PGA

γ-PGA can exist in different conformational states. Depending on the environmental conditions, γ-PGA can exhibit five different conformations: α-helix, β-sheet, helix-to-random coil transition, random coil and enveloped aggregate (Ho et al., 2006). The conformational state of γ-PGA can change depending on a number of factors, such as pH, polymer concentration and ionic strength. It has been shown that γ-PGA purified from B. licheniformis can exist in different conformational states depending on the concentration of γ-PGA and the pH of the solution (He et al., 2000). At low concentration (0.1 % w/v) and pH<7.0, PGA adopts a conformation based largely on α-helices, whereas a β-sheet-based conformation predominates at higher pH (Bhat, 2012). The β-sheet conformation seems to expose the negative charges of γ-PGA very efficiently. γ-PGA conformation is sensitive to small changes in specific factors, e.g. changes in PGA side-chain ionization can have a pronounced effect on the conformation (Shih & Van, 2001; Tiffany & Krimm, 1969; Bhat, 2012).

γ-PGA also varies in enantiomeric composition (Table 1). The enantiomeric composition of γ-PGA determines how γ-PGA is extracted after fermentation. If γ-PGA contains only L or D enantiomers, then it dissolves in ethanol. However, if L and D forms are in equimolar amounts, then γ-PGA precipitates in ethanol (Candela & Fouet, 2006).

Optimization to determine the effect of the enantiomeric composition of γ-PGA on its behaviour and efficacy for a specific application is necessary. When γ-PGA with different enantiomeric compositions was tested for its antifreeze properties, it was seen that the antifreeze activity of γ-PGA was not affected by its enantiomeric composition, but only by its molecular mass (Shih et al., 2003). The antifreeze property was seen to be affected by the concentration of cations in the order of Mg²⁺ ≫ Ca²⁺ ≈ Na⁺ ≫ K⁺ (Bhat, 2012).

Culture conditions seem to have an impact on the enantiomeric composition and molecular mass of γ-PGA, both of which can alter its properties (Rehm, 2009). It would be interesting to see how these properties affect specific applications. Knowledge of the conformational state of γ-PGA is therefore essential when it is used in medical/food applications as it is known that a small change in environmental conditions can change the properties of γ-PGA to a large extent.

Production of γ-PGA by microbial fermentation

Much research has gone into the production of γ-PGA from bacterial fermentation. When α-PGA is produced synthetically, the product has a molecular mass of <10 kDa, which limits its application. However, when PGA is produced from bacteria (γ-PGA), it has a molecular mass >10 kDa and usually ranges from ~100 to >1000 kDa (Richard & Margaritis, 2003; Park et al., 2005; Bajaj et al., 2009).

Although the microbial production of γ-PGA has since been established, the cost of production, which ultimately affects the market price (£137 per 100 mg high-purity sodium salt γ-PGA; Sigma Aldrich) of the polymer, is presently very high and this is a major limitation to the widespread application of the polymer. Based on this, most research on microbial production of γ-PGA is focused on the optimization of growth conditions with the potential to produce a high yield, specific enantiomeric composition and desired molecular mass of γ-PGA at reduced cost. The medium used to produce γ-PGA by various bacteria is crucial because it directly affects the properties of γ-PGA. It has been shown, for instance, that different concentrations of NaCl in the medium yield γ-PGA with different molecular masses (Birrer et al., 1994). Birrer et al. (1994) also studied the effect of ionic strength on γ-PGA production by B. licheniformis ATCC 9945a. When the NaCl concentration in the medium was increased from 0 to 4 %, the molecular mass of the resultant γ-PGA increased
by a factor of 1.8 (from $1.2 \times 10^6$ to $2.2 \times 10^6$ Da). High-
molecular-mass $\gamma$-PGA is very promising for various industrial applications. However, it is generally diverse in its molecular structure, and during the fermentation it is co-produced with different polysaccharides and other biopolymers. Production of $\gamma$-PGA from *B. subtilis* (natto) with a molecular mass $>2 \times 10^6$ Da has proved to be challenging for various reasons: $\gamma$-PGA synthesis or elongation is sometimes coupled with degradation of $\gamma$-PGA towards the later stages of fermentation and the $\gamma$-PGA synthase complex itself is not stable. However, *B. subtilis* (chungkookjang) cultivated in a medium with a high concentration of ammonium sulphate has been found to produce super-high-molecular-mass $\gamma$-PGA without the presence of any byproducts and was richer in $\delta$ enantiomer. Park *et al.* (2005) also managed to isolate $\gamma$-PGA which had a molecular mass $>2 \times 10^6$ Da, but it was difficult to accurately measure such a high-molecular-mass $\gamma$-PGA. It was thought to be $\sim 7 \times 10^6$ Da.

According to Shih & Van (2001), $\gamma$-PGA-producing bacteria have been divided into two groups depending upon their nutrient requirement for $\gamma$-PGA production — those that require L-glutamic acid in the medium and those that do not require L-glutamic acid. The L-glutamic-acid-dependent bacteria include *B. subtilis* (chungkookjang) (Ashiuchi *et al.*, 2001a), *B. licheniformis* 9945a (Birrer *et al.*, 1994) *B. subtilis* CGMCC 0833 (Wu *et al.*, 2010), *B. licheniformis* NK-03 (Cao *et al.*, 2010) and *B. subtilis* (natto) ATCC 15245 (Bhat *et al.*, 2013), whilst the non-L-glutamic-acid-dependent producers include *B. subtilis* C1 (Shih *et al.*, 2005), *B. amyloliquefaciens* LL3 (Cao *et al.*, 2011) and *B. subtilis* C10 (Zhang *et al.*, 2012). For L-glutamic-acid-dependent bacteria, the PGA yield increases with an increase in the L-glutamic acid concentration in the medium. However, these bacteria can produce $\gamma$-PGA even in the absence of exogenously supplied L-glutamic acid due to the resource of L-glutamic acid obtained through the *de novo* pathway (Kunioka & Goto, 1994; Buescher & Margaritis, 2007). The L-glutamate-independent producers are more desirable for industrial $\gamma$-PGA production than the glutamate-dependent producers due to their low cost of production and simple fermentation process (Cao *et al.*, 2011). However, their industrial utilization is limited due to lower $\gamma$-PGA productivity compared with those of L-glutamate-dependent producers. This has led to the development of genetically engineering non-glutamate-dependent producers such as *B. amyloyliquefaciens* NK-1 (Feng *et al.*, 2014) as well as laboratory strains such as *B. subtilis* MA41 (Ashiuchi *et al.*, 2006), *E. coli* (Cao *et al.*, 2013) and *B. subtilis* 168 (Scoffone *et al.*, 2013) for high $\gamma$-PGA productivity.

### Production with Bacillus species

**B. licheniformis**

*B. licheniformis* has been widely used for the production of $\gamma$-PGA. *B. licheniformis* 9945a (NCIM 2324) is a prominent strain that has been used for $\gamma$-PGA production. As mentioned earlier, efforts have been made to optimize production of $\gamma$-PGA with the aim to obtain maximum yield. Bajaj *et al.* (2008) enhanced the production of $\gamma$-PGA using *B. licheniformis* NCIM 2324 in solid-state fermentation. Using the ‘one factor at a time’ method, they investigated the effect of solid substrates, moisture content, pH, carbon and nitrogen sources, amino acids, and TCA cycle intermediates on the production of $\gamma$-PGA. Their optimized media gave a maximum $\gamma$-PGA yield of 98.64 mg (g dry solids)$^{-1}$ in solid fermentation.

Bajaj *et al.* (2009) also optimized the production of $\gamma$-PGA with *B. licheniformis* NCIM 2324 using the ‘one factor at a time’ method. The optimum nutrient concentrations were devised with the help of response surface methodology. These were then tested experimentally. The yield obtained

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**Table 1. Enantiomeric composition of PGA-producing organisms**

<table>
<thead>
<tr>
<th>$\gamma$-PGA producer</th>
<th>PGA composition (%)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$D$-Glutamate</td>
<td>$L$-Glutamate</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em></td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> ATCC 9945a (now <em>Bacillus licheniformis</em> 9945a)</td>
<td>$-10$–100*</td>
<td>$-10$–90*</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td><em>Bacillus chungkookjang</em></td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td><em>Staphylococcus</em> epidermidis</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td><em>Natrialba aegyptiaca</em></td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> BS 62</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

*Dependent on the concentration of Mn$^{2+}$.*
with the devised medium (in g l\(^{-1}\): glycerol, 62.4; citric acid, 15.2; ammonium sulphate, 8.0; \(\\) L-glutamic acid, 20) was 26.12 g l\(^{-1}\) as opposed to the basal medium (5.27 g l\(^{-1}\)). The molecular mass of \(\gamma\)-PGA thus obtained was 2.1 \times 10^{5} \text{ Da}.

Based on the above work, Bajaj &Singhal (2009) developed a process that achieved a productivity of 35.75 g \(\gamma\)-PGA l\(^{-1}\) using \(B.\) licheniformis NCIM 2324. This is the highest productivity of \(\gamma\)-PGA obtained with the help of any strain of \(B.\) licheniformis in submerged fermentation available thus far. This was achieved by feeding \(B.\) licheniformis NCIM 2324 with 0.07 g L-glutamine l\(^{-1}\) and 1.46 g \(\alpha\)-ketoglutaric acid l\(^{-1}\) (which are metabolic precursors for \(\gamma\)-PGA production), in addition to the basal medium. The yield of \(\gamma\)-PGA of 35.75 g l\(^{-1}\) was in contrast to 26.12 g l\(^{-1}\) when no metabolic precursors were added. The added precursors helped in the better utilization of \(\) L-glutamic acid.

\(B.\) licheniformis A13, an exogenous glutamate-independent producer, presented a \(\gamma\)-PGA yield of 28.2 g l\(^{-1}\) in an optimized medium (g l\(^{-1}\): glucose, 50; NH\(_{4}\)Cl, 3; yeast extract, 2; MgSO\(_{4}\).7H\(_{2}\)O, 0.8; NaCl, 0.8; CaCl\(_{2}\).2H\(_{2}\)O, 0.00084; K\(_{2}\)HPO\(_{4}\), 6.4; FeSO\(_{4}\).4H\(_{2}\)O, 0.006; 0.1 ml trace element solution and 25 ml culture volume) devised using the Plackett–Burman design after 72 h of inoculation (Mabrouk et al., 2012). Results showed that yeast extract and medium volume were the two factors affecting \(\gamma\)-PGA production.

The Plackett–Burman experimental design was also used to evaluate the culture requirements for \(B.\) licheniformis SAB-26 (Soliman et al., 2005). Fifteen variables were tested for their effect on \(\gamma\)-PGA production, of which K\(_{2}\)HPO\(_{4}\), K\(_{2}\)HPO\(_{4}\), (NH\(_{4}\))\(_{2}\)SO\(_{4}\) and casein hydrolysate were the most significant. When L-glutamic acid was used as the nitrogen source, the production of \(\gamma\)-PGA was repressed. Hence, \(B.\) licheniformis SAB-26 could be classified as a glutamate-independent \(\gamma\)-PGA producer. A yield of 33.5 g l\(^{-1}\) was obtained when the bacteria were grown on the optimized medium, which was three times more than when grown on basal medium.

\(B.\) subtilis

Bovarnich (1942) first showed that \(\gamma\)-PGA is freely secreted into the medium after fermentation of \(B.\) subtilis. Following this, considerable research has been carried out into the production of \(\gamma\)-PGA using \(B.\) subtilis. More strains of \(B.\) subtilis have been investigated for the production of \(\gamma\)-PGA than of \(B.\) licheniformis.

\(\gamma\)-PGA production was recently evaluated by Soffone et al. (2013). This was done by knocking out \(pgdS\) and \(ggt\) (genes for two important \(\gamma\)-PGA-degrading enzymes) in the laboratory strain \(B.\) subtilis 168. The effects of single mutations (either \(pgdS\) deletion or \(ggt\) deletion) and double mutation (both \(pgdS\) and \(ggt\) deletion) on \(\gamma\)-PGA production were assessed. Results revealed that single mutations had no significant improvement on \(\gamma\)-PGA yield, whereas a twofold increase (>40 g l\(^{-1}\)) was observed in the double-mutant strain compared with the WT strain. However, number average molecular mass and weight average molecular mass of \(\gamma\)-PGA produced by double-mutant strains were lower compared with those of single-mutant strains and the WT strain. The \(pgdS\) mutant strain presented the highest molecular mass (>3 \times 10^{8} \text{ Da}) probably due to reduced \(\) endo-degradation activities.

High-yield, cost-effective and large-scale production of \(\gamma\)-PGA from \(B.\) subtilis ZJU-7 \((B.\) subtilis CGMCC1250\) has been reported by Huang et al. (2011). Their findings showed that 40 g yeast extract l\(^{-1}\), 30 g \(\) L-glutamate l\(^{-1}\) and 20 g initial glucose l\(^{-1}\) as well as keeping the glucose concentration in the range of 3–10 g l\(^{-1}\) through a fed-batch approach greatly improved the production of \(\gamma\)-PGA 1.4- to 3.2-fold compared with that in batch fermentation. An overall \(\gamma\)-PGA concentration of 101.1 g l\(^{-1}\) and a productivity of 2.19 g l\(^{-1}\) were recorded.

Solid-state fermentation of \(B.\) subtilis CCTCC202048 was optimized for the production of \(\gamma\)-PGA by lian et al. (2005). Maximum \(\gamma\)-PGA production [83.61 g (kg dry solids\(^{-1}\))] was obtained when both soybean cake powder and wheat bran were used as mixed substrates in a ratio of 11:9 (w/w) in addition to glutamate (40.14 g kg\(^{-1}\)), citric acid (18.50 g kg\(^{-1}\)), NH\(_{4}\)NO\(_{3}\) (20.05 g kg\(^{-1}\)) and mineral salts (MgSO\(_{4}\).7H\(_{2}\)O, CaCl\(_{2}\).2H\(_{2}\)O, FeCl\(_{3}\).6H\(_{2}\)O and MnSO\(_{4}\).H\(_{2}\)O). Solid-state fermentation could prove to be useful for the large-scale production of \(\gamma\)-PGA owing to high productivity and low production costs.

Shih et al. (2005) produced a novel glycerol-\(\gamma\)-PGA derivative using \(B.\) subtilis C1 without L-glutamate in the medium (Shih et al., 2005). \(B.\) subtilis C1 was dependent on citric acid and glycerol. If either of these was absent in the medium, production of \(\gamma\)-PGA was negligible. The molecular mass of the conjugate \((1 \times 10^{7} \text{ Da})\) was higher than the super-high-molecular mass \(\gamma\)-PGA produced by Park et al. (2005) owing to the presence of glycerol. The ratio of \(\gamma\)-PGA to glycerol in the conjugate was 10:1. The conjugate had a higher concentration of \(d\)-glutamic acid units (97 %) than \(L\)-glutamic acid units. Interestingly, Mn\(^{2+}\) did not seem to affect the enantiomeric composition of the glycerol-\(\gamma\)-PGA conjugate.

In contrast, Wu et al. (2006) showed that Mn\(^{2+}\) affected the stereochemical and enantiomeric composition of \(\gamma\)-PGA produced with \(B.\) subtilis NX-2. When the concentration of Mn\(^{2+}\) was varied from 0 to 0.09 g l\(^{-1}\), the proportion of \(d\)-glutamate increased from 18 to 77 %. Mn\(^{2+}\) seemed to affect the stereochemical properties of \(\gamma\)-PGA by altering the activity of glutamate racemase.

Other bacteria

The \(\gamma\)-PGA synthase genes \(pgsBCA\) and \(racE\) from an \(L\)-glutamate-dependent \(\gamma\)-PGA producer \(B.\) licheniformis NK-03 and a non-\(L\)-glutamate-dependent \(\gamma\)-PGA producer \(B.\)
Amyloliquefaciens LL3 were cloned and co-expressed in E. coli JM109 for evaluation of γ-PGA productivity (Cao et al., 2013). The results showed that pgsB and pgsC of both strains are highly similar with 93.13 and 93.96% resemblance, whilst the pgsA and racE presented 78.53 and 84.5% similarity, respectively. The results further showed that the four engineered strains primarily produced γ-PGA in both glucose and L-glutamate medium after culturing for 24 h. The PgsBCA developed from B. amyloliquefaciens LL3 seemed to possess better catalytic activity than that of B. licheniformis NK-03 irrespective of the harbouring vector as the quantity of γ-PGA produced by B. amyloliquefaciens LL3-pgsBCA was higher than that by B. licheniformis NK-03-pgsBCA. There was significant improvement in productivity and D-isomer content of γ-PGA from B. amyloliquefaciens LL3-derived PgsBCA and RacE and B. licheniformis NK-03-derived PgsBCA and RacE than in γ-PGA from B. amyloliquefaciens LL3-derived PgsBCA and B. licheniformis NK-03-derived PgsBCA, showing that the incorporation of racE enhanced the productivity of γ-PGA as well as its D-isomer.

In order to avoid the addition of exogenous L-glutamic acid, B. subtilis was co-cultured with Corynebacterium glutamicum using glucose and sucrose as a mixed carbon source, and this turned out to be helpful in reducing the fermentation time and production cost (Xu et al., 2005). The average molecular mass of γ-PGA thus produced was 1.24 × 10^6 Da.

B. anthracis is known to produce a pure D enantiomer of γ-PGA (Zwartouw & Smith, 1956). The prominent difference in the mechanism of production of B. anthracis compared with other Bacillus species is that it does not secrete γ-PGA into the medium, but instead, it is peptidoglycan bound. This makes the recovery and purification procedure (which involves autoclaving and autolysis of cells) tedious. More importantly, industrial production of γ-PGA from B. anthracis is not viable owing to its toxicity. In fact, the anchored γ-PGA is responsible for the non-immunogenic capsule of B. anthracis, which has been associated with the lethal toxin (Ezzell et al., 2009). Hence, to render B. anthracis immunogenic, its cap gene responsible for the anchoring of γ-PGA onto its surface needs to be targeted (Candela & Fouet, 2006).

It was shown that Bacillus thuringiensis sv. Monterrey strain BGSC 4A1 produced a γ-PGA capsule similar to that of B. anthracis (Cachat et al., 2008). B. thuringiensis sv. Monterrey strain BGSC 4A1 and B. anthracis (Ames) had four common alleles, gmk-1, pta-1, pur-1 and tpi-1, whilst the three other alleles, glpF-57, ilvd-52 and pycA-52, differed by 2, 2 and 3 nt respectively. The γ-D-PGA-producing genes bear similarity with those of B. anthracis and are present on a plasmid (pA1J-1). Discovery of a γ-PGA capsule in this strain of B. thuringiensis could indicate the ability of the bacteria to be pathogenic under certain conditions.

The indispensable pgsBCA complex was also introduced into tobacco leaves via Agrobacterium infection (Tarui et al., 2005). γ-PGA production was only seen in plant tissue where all three pgsBCA genes transferred successfully and 600 μg γ-PGA (g leaf material)^-1 was obtained.

Characterization of γ-PGA

Molecular characterization of γ-PGA is essential in order to evaluate its properties and hence its potential areas of application.

Molecular mass determination

Gel permeation chromatography (GPC) is the most commonly used method for determining the molecular mass of γ-PGA. GPC uses a range of mobile phases and calibrates against standards of diverse molecular masses (Birrer et al., 1994). The molecular mass of γ-PGA is important for understanding its function (Sung et al., 2005b). The molecular mass of γ-PGA produced by Bacillus species ranges from 10^5 to 10^6 Da (Shih & Van, 2001) and differs depending on the application for which it is required. Molecular mass reduction can be an important step in the production of γ-PGA for drug delivery applications. Different techniques used for this purpose include ultrasonic degradation, alkaline hydrolysis, alteration of medium composition and microbial or enzymic degradation (Shih & Van, 2001). Ultrasonic degradation in particular was shown to be an effective method to reduce both the molecular mass and the dispersity of naturally produced PGA without disturbing the chemical constitution of the polymer (Graciela et al., 2000). Richard & Margaritis (2006) carried out in situ depolymerization of γ-PGA in the cell-free fermentation broth of B. subtilis IFO 3335. The molecular mass, when measured using GPC and intrinsic viscosity correlations, reduced from ~4 × 10^6 to 5.5 × 10^5 Da over a period of 144 h. As with the study of Yao et al. (2009), the dispersity of γ-PGA decreased as a function of hydrolysis time. Enzymic degradation seems to be a better method to obtain γ-PGA of the required molecular mass in a controlled fashion.

Amino acid analysis

The detection of only glutamic acid in amino acid and TLC analysis indicates the purity of γ-PGA (Shih & Van, 2001). For amino acid analysis of γ-PGA, purified γ-PGA is hydrolysed with 6 M HCl at 100 °C for a number of hours (depending on the amount of γ-PGA) in an airtight tube, followed by the removal of residual HCl by evaporation and then the hydrolysed product is dissolved in distilled water before analysing the amino acid contents by TLC (Yokoi et al., 1995). Shih et al. (2001) purified γ-PGA by hydrolysis with 6 M HCl at 110 °C for 24 h in a closed and evacuated tube, and analysed the product using an amino acid analyser. TLC was then carried out on a cellulose plate with solvent systems of butanol/acetic acid/water (3:1:1, w/w/w) and 96 % ethanol/water (63:37, w/w), and amino acids were identified by spraying with 0.2 % ninhydrin in acetone (Yokoi et al., 1995).
NMR spectroscopy

1H- and 13C-NMR spectroscopy are usually performed to determine the homogeneity and degree of esterification of γ-PGA (Birrer et al., 1994; Borbely et al., 1994). Chemical shifts from resulting NMR spectra are measured relative to known standards.

Fourier transform-IR spectroscopy

The Fourier transform-IR spectroscopy technique which employs an IR absorption spectrum is used for product identification. The method produces IR spectra of γ-PGA with peaks corresponding to specific bonds in the compound. According to Ho et al. (2006), the IR spectra of γ-PGA (free acid form) and γ-polyglutamate salts in KBr pellets indicate distinctive strong amide absorption at ~1620–1655 cm⁻¹, a weaker carbonyl C=O absorption at ~1394–1454 cm⁻¹, a strong hydroxyl OH absorption at ~3400–3450 cm⁻¹ and a characteristic strong C-N groups absorption in the range from 1085 to 1165 cm⁻¹. The absorption peaks between 2900 and 2800 cm⁻¹ are characteristic of aliphatic N-H stretching, whilst those around 1600–1660 and 1390–1450 cm⁻¹ exhibit characteristics of amide groups and C=O groups, respectively.

Applications of γ-PGA

γ-PGA is an extremely important substance that has been exploited for a wide array of useful applications due to its unique properties. It is biodegradable, edible and non-toxic for humans, which itself is a prerequisite for being used for human benefit.

Medical applications

This section will describe some important potential medical applications that have exploited γ-PGA. Optimization of γ-PGA for use in drug delivery applications is a crucial step in making this technology applicable. Molecular mass has been found to be a decisive factor on which the drug delivery properties of γ-PGA depend. Polymers of different molecular masses are required to control a drug’s release into tissue. In this regard, it was found that the molecular mass of γ-PGA is often higher than that required for drug delivery applications (Richard & Margaritis, 2006). The molecular mass of γ-PGA required for conjugation with the drug paclitaxel (Taxol), for example, has been determined to be 3 × 10⁴–6 × 10⁶ Da (Li et al., 1998). Li et al. (1998) designed a PGA–paclitaxel conjugate by covalent bonding of natural paclitaxel to PGA in order to improve the stability and antitumour efficiency of paclitaxel, and to offer a water-soluble paclitaxel. The PGA–paclitaxel conjugate showed significantly better antitumour activity against murine and human tumour xenografts, including breast and ovarian cancers in animal models, than did regular paclitaxel. This was thought to be due to the uptake of the PGA–paclitaxel conjugate by tumour cells being about fivefold greater than that of paclitaxel when comparable doses were used. In contrast to paclitaxel, in vitro studies with the PGA–paclitaxel conjugate showed that it supports neither tubulin polymerization nor the growth and survival of a Taxol-dependent CHO cell line (Li et al., 1998).

Singer (2005) also devised a conjugate of γ-PGA and paclitaxel called paclitaxel poliglumex. Paclitaxel poliglumex is a macromolecular taxane designed to improve the safety and efficiency of paclitaxel by enhancing its pharmacokinetic profile as well as offering a water-soluble substitute for the standard paclitaxel design. Preclinical developments in animal tumour models showed that paclitaxel poliglumex is as effective as regular paclitaxel and is associated with longer tumour exposure to active drug and systemic exposure is reduced. Paclitaxel poliglumex presents a number of advantages over standard paclitaxel, including solubility in water, stability in plasma that relates to a reduced systemic exposure to free paclitaxel, smaller volume of distribution, longer distribution and elimination phases of paclitaxel poliglumex’s pharmacological profile, and increased tumour selectivity via enhanced accumulation and retention in tumour tissue. In contrast to non-degradable polymers, l-glutamic acid, the catabolic product of γ-PGA, can enter normal cellular metabolism and is not excreted by the kidney (Singer, 2005).

Kim et al. (2007) have shown that administering γ-PGA with a molecular mass as high as 2 × 10⁶ Da orally induces significant natural killer (NK)-cell-mediated antitumour immunity in mice bearing major histocompatibility complex class I-deficient tumours (Kim et al., 2007). The mechanism underlying the antitumour immunity was seen to be due to the activation of NK cells by γ-PGA rather than a direct cytotoxic effect. Their results showed that γ-PGA (2 × 10⁶ Da) is as good as or a better stimulator of antitumour effects against B16 tumours in C57BL/6 mice than β-glucan (curdlan) – an immunomodulating antitumour agent known to activate NK cells. This research suggests the potential use of γ-PGA in cancer immunotherapy.

Ryu et al. (2011) developed nanoparticles (NPs) composed of γ-PGA coupled with l-phenylalanine (γ-PGA–Phe NPs). They explored the possibility of these γ-PGA–Phe NPs both in vitro and in vivo in the treatment of retinal diseases. γ-PGA–Phe NPs with Texas red-labelled ovalbumin were used to investigate the dynamics of NPs in the eye, whilst those containing dexamethasone were used to investigate their potential in in vivo immunosuppressive treatment of macrophages and microglia in several pathological retina disorders (Ryu et al., 2011). Results suggested that γ-PGA-Phe NPs are suitable, particularly under pathological conditions, for regulating inflammatory phagocytic cells in the retina. Additionally, results revealed that γ-PGA-Phe NPs can be used as a carrier for long-term drug delivery in the damaged retina. γ-PGA-Phe NPs possess important advantages over steroids such as triamcinolone acetae,
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<th>Field</th>
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<tr>
<td>Wastewater treatment</td>
<td>Biopolymer flocculant</td>
<td>γ-PGA produced by <em>B. subtilis</em> R 23 showed a high flocculating activity that could be further enhanced by the addition of cations.</td>
<td>Bajaj &amp; Singhal (2011)</td>
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<td>Efficient flocculation of various organic and inorganic compounds was demonstrated by γ-PGA produced by <em>B. licheniformis</em> CCRC 12826.</td>
<td>Shih <em>et al.</em> (2001)</td>
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<td>Heavy metal removal</td>
<td>γ-PGA covalently incorporated into microfiltration membranes via attachment to their membrane pore surfaces exhibited super-high heavy metal sorption ability.</td>
<td>Bhattacharyya <em>et al.</em> (1998)</td>
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<td>γ-PGA was found to bind and efficiently remove &gt;99.8% of lead ions from water via a suitable low-pressure ultrafiltration technique.</td>
<td>Hajdu <em>et al.</em> (2012)</td>
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<td>Dye removal</td>
<td>γ-PGA (9.9 × 10^5 Da) could be used effectively to remove basic dyes from aqueous solution. It was found that 98% of the dye adsorbed on γ-PGA could be recovered at pH 1, which facilitates the reuse of spent γ-PGA.</td>
<td>Inbaraj <em>et al.</em> (2006)</td>
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<td>Medicine</td>
<td>γ-PGA-coated super paramagnetic iron oxide NPs demonstrated high heavy metal removal efficiency from simulated gastrointestinal fluid and a metal solution.</td>
<td>Inbaraj &amp; Chen (2012)</td>
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<td>Drug carrier/deliverer</td>
<td>γ-PGA with covalently attached cisplatin was shown to reduce the toxicity of cisplatin, whilst efficiently decreasing the tumour size of xenografted human breast tumours in nude mice as well as lengthen the survival of nude mice grafted with Bcap-37 tumour cells.</td>
<td>Ye <em>et al.</em> (2006)</td>
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<td>A macromolecular conjugate of paclitaxel and γ-L-PGA called paclitaxel poliglumex exhibited outstanding benefits over conventional paclitaxel. Paclitaxel poliglumex was accumulated in tumour tissue, where it gradually discharged the active agent paclitaxel.</td>
<td>Singer (2005)</td>
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<td>Tissue engineering</td>
<td>A γ-PGA/chitosan composite biomaterial demonstrated potential application in tissue engineering as it is more hydrophilic and cytotocompatible than conventional chitosan matrices.</td>
<td>Hsieh <em>et al.</em> (2005)</td>
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<td>A PEC of chitosan and γ-PGA demonstrated potential application in wound dressing. The complex presented sufficient moisture content and showed good mechanical properties, which would allow the dressing to be easily removed from the wound surface without destroying renewed tissues.</td>
<td>Tsao <em>et al.</em> (2011)</td>
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<td>Biological adhesive</td>
<td>A mixture of gelatin and γ-PGA aqueous solution which resulted in the formation of a hydrogel in the presence of water-soluble carbodiimide demonstrated better lung adhesion and air-leak sealing than conventional fibrin glue.</td>
<td>Otani <em>et al.</em> (1999)</td>
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<td>Calcium absorption</td>
<td>Administration of γ-PGA increased calcium absorption in the intestine in post-menopausal women by inhibition of the formation of an insoluble calcium complex with phosphate. Can be potentially used for treatment of bone disorders.</td>
<td>Tanimoto <em>et al.</em> (2007)</td>
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<td>Food industry</td>
<td><em>Natto</em> mucilage containing γ-PGA greatly improved calcium solubility in vitro and in vivo in rats as well as the calcium content of their bones.</td>
<td>Tanimoto <em>et al.</em> (2001), <em>Ho et al.</em> (2008)</td>
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<td>Food supplement/osteoporosis-preventing agent</td>
<td>The addition of γ-PGA to wheat bread reduced its hardness through storage. γ-PGA also enhanced the rheological and thermal properties of wheat dough. γ-PGA was demonstrated to improve the texture of sponge cake.</td>
<td>Shyu <em>et al.</em> (2008), Shyu &amp; Sung (2010)</td>
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<td>Texture enhancer</td>
<td>γ-PGA was demonstrated to reduce oil uptake during deep-fat frying. γ-PGA added to doughnuts showed a fivefold decrease in the amount of oil absorbed compared with normal doughnuts. The overall appearance and taste of the product was better than those of normal doughnuts.</td>
<td>Lim <em>et al.</em> (2012)</td>
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<td>Oil-reducing agent</td>
<td>γ-PGAs with molecular masses &lt;20 kDa were demonstrated to have higher antifreeze activities than high antifreeze agents like glucose and they do not interfere with the taste of foods.</td>
<td>Mitsuiki <em>et al.</em> (1998)</td>
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<td>Cryoprotectant</td>
<td>γ-PGA with a molecular mass of 257 kDa has been used as a cryoprotectant for probiotic bacteria to improve their survival during production.</td>
<td>Bhat <em>et al.</em> (2013)</td>
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<td>Bitterness-relieving agent</td>
<td>γ-PGA has been used as a bitterness-relieving agent.</td>
<td>Sakai <em>et al.</em> (2000)</td>
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which has been used to effectively treat retinal disorders. These advantages include lack of complications of steroid-induced glaucoma and post-capsular cataract formation. Unlike steroids, where direct application is toxic for retinal neurons, γ-PGA-Phe NPs specifically target macrophages and microglia, thereby minimizing steroid complications (Ryu et al., 2011).

A polyelectrolyte complex (PEC) comprising chitosan as a cationic polyelectrolyte and γ-PGA as an anionic polyelectrolyte was designed by Tsao et al. (2011) for wound dressing. Results showed that the chitosan/γ-PGA PECs exhibit adequate moisture content, with γ-PGA reducing the risk of dehydration compared with regular chitosan. Preclinical studies in animal models showed that wounds treated with the chitosan/γ-PGA PECs healed faster than wounds given no treatment. Chitosan/γ-PGA PECs showed excellent suppression of inflammatory cells in contrast to neat chitosan, indicating that γ-PGA has an anti-inflammatory effect. Keratin development was more assimilated in those wounds treated with chitosan/γ-PGA PECs compared with the neat chitosan or control. Chitosan/γ-PGA PECs were also easily taken off the wound surface after healing without causing damage to the newly regenerated tissue, suggesting that chitosan/γ-PGA PECs are potential materials for wound dressings.

The suppressive effect of γ-PGA on the SOS response of *Salmonella typhimurium* induced by several direct and indirect mutagens has been studied and evaluated (Sato et al., 2008). The sodium salt of γ-PGA was used with a range of molecular masses: 50, 2000, 4000, 6000 and 8000 kDa. When 3% Na·γ-PGA with a molecular mass of 4000 kDa was used, 80–90% suppression was observed irrespective of the type of chemical mutagen, and this was better than carboxymethylcellulose (20–40%) and xanthan gum (40–75%). The antimutagenic property of γ-PGA was shown to be dependent on the molecular mass. γ-PGAs with molecular masses of 50 and >6000 kDa did not show any distinct antimutagenic property. It is believed that after appropriate tests, γ-PGA could be used as an antimutagenic food additive.

### Food applications

One of the naturally occurring sources of γ-PGA is the mucilage of *natto* (fermented soybeans), which is a traditional food in Japan. As it is food derived, γ-PGA can be used for food applications.

The effect of γ-PGA on the viability of probiotic bacteria during freeze-drying was investigated by Bhat et al. (2013). Results showed that 10% γ-PGA was found to protect
Lactobacillus paracasei significantly better than 10 % sucrose, which has been reported previously to offer better protection during freeze-drying of lactobacilli compared with trehalose and sorbitol (Siaterlis et al., 2009), and nata, a bacterial cellulose produced by Acetobacter xylinum (Jagannath et al., 2010).

Lim et al. (2012) investigated the effect of γ-PGA on oil absorption and moisture loss in doughnuts during deep-fat frying. It was shown that at a frying time of 4 min and γ-PGA concentration of 1 g (100 g dough)⁻¹, oil uptake was reduced by fivefold in doughnuts containing γ-PGA [0.2 g (g dough)⁻¹] compared with regular doughnuts [0.7 g (g dough)⁻¹]. Overall, γ-PGA doughnuts had better appearance and taste than regular doughnuts. γ-PGA is therefore a potential oil-reducing agent in deep-fat fried foods.

The effects of γ-PGA on the viscosity, foam stability and emulsifying properties of sponge cake batter at different concentrations (0.05, 0.1 and 0.5 g kg⁻¹, w/w) were explored by Shyu & Sung (2010). The addition of 0.5 g γ-PGA kg⁻¹ to sponge cake batter showed an increase in viscosity, foam stability and emulsion stability. Sponge cake hardness and chewiness were also decreased during storage with the addition of γ-PGA.

γ-PGA was found to increase calcium absorption in vitro and in vivo, and decrease bone loss in humans (Tanimoto et al., 2007). γ-PGA increased the bioavailability of calcium by increasing its solubility and intestinal absorption. The increase in calcium solubility was as a result of the inhibition of the production of insoluble calcium phosphate (Tanimoto et al., 2007). Post-menopausal women who were given a single dose of γ-PGA were seen to have better intestinal absorption. Individuals with lower basal absorptive capacity were particularly seen to benefit from γ-PGA.

Wastewater treatment

Chang et al. (2013) investigated the capability of γ-PGA-modified superparamagnetic iron oxide NPs (γ-PGA/Fe₃O₄ NPs) in the removal of heavy metal ions including Cr³⁺, Cu²⁺, Pb²⁺ and Ni²⁺. The γ-PGA/Fe₃O₄ NPs showed outstanding removal activity for all metals and better removal efficiency than either Fe₃O₄ NPs or γ-PGA only, whilst overcoming the weaknesses of using either Fe₃O₄ NPs or γ-PGA on their own. This indicates that γ-PGA has potential applications in wastewater treatment.

γ-PGA (5.8 × 10⁶ Da) produced from B. subtilis 2063 showed great flocculating activity indicating that γ-PGA can be a potential alternative to conventional synthetic flocculants (known to persist in the environment) in wastewater treatment (Bhunia et al., 2012). Similarly, Bajaj & Singhal (2011) studied the flocculation properties of γ-PGA (6.2 × 10⁶ Da) derived from B. subtilis R 23. Results indicated that γ-PGA is an excellent flocculant, and can be used in wastewater treatment plants and downstream processing of food and fermentation industries.

Bodnár et al. (2008) have also investigated the preparation and characterization of novel biodegradable NPs based on complexation of γ-PGA derived from B. licheniformis 9945a with bivalent lead ion. The prepared complex was stable in aqueous media under low, neutral and mild alkaline conditions. This complex is thought to have useful water treatment applications due to its biodegradability along with heavy metal-binding activity.

γ-PGA (9.9 × 10⁵ Da) can be used effectively to remove basic dyes from aqueous solution (Inbaraj et al., 2006). It was found that 98% of the dye adsorbed on γ-PGA could be recovered at pH 1, which facilitates the re-use of spent γ-PGA. As γ-PGA is non-toxic and biodegradable, the adsorption system developed could offer eco-friendly solutions to the dye industry.

Other applications

The applications of γ-PGA in skincare products were explored by Ben-Zur & Goldman (2007). They found that γ-PGA is a good hydrophilic humectant and has the ability to increase the production of natural moisturizing factors such as urocanic acid, pyrrolidone carboxylic acid and lactic acid compared with hyaluronic acid and soluble collagen. γ-PGA has also been shown to enhance the elasticity of the skin more than collagen and hyaluronic acid as well as refresh and nourish the skin, making it smoother and more desirable (Ben-Zur & Goldman, 2007).

γ-PGA has been used successfully as an active ingredient in a hyaluronidase inhibitor (Sung et al., 2005a). Hyaluronidase is an enzyme that degrades hyaluronic acid present in the skin dermis. The composition was tested on 50 women (aged 30–50 years), and was shown to maintain skin elasticity by inhibiting the activity of hyaluronidase and reduce allergic reactions by inhibiting the permeability of inflammatory cells.

Hu et al. (2008) tested whether γ-PGA could be used to induce protein crystallization in easily crystallizable proteins (lysozyme, glucose isomerase and xylanase). Both low-molecular-mass γ-PGA (Na⁺ salt: 2 × 10⁵–4 × 10⁵ Da) and high-molecular-mass γ-PGA (Na⁺ salt: >1 × 10⁶ Da) were tested, and results showed that γ-PGA could indeed induce precipitation in proteins as additives or stand-alone precipitants.

The number of potential application for γ-PGA is vast and still increasing. Table 2 attempts to summarize the diversity of γ-PGA uses within a range of application areas.

Conclusion

γ-PGA is biodegradable, edible and non-immunogenic, and can therefore be used safely in a variety of applications that are increasing rapidly. Knowledge of the genes and enzymes involved in γ-PGA regulation throws light on the conditions that support γ-PGA production (e.g. high osmolarity), and this information could be used to isolate...
and screen new γ-PGA-producing strains from different sources. As different applications of γ-PGA exploit different specific properties of γ-PGA, it would be interesting to analyse the properties of the γ-PGA that would be produced by different bacteria grown under a range of culture conditions.

For conventional materials to be replaced with γ-PGA, the cost of production of γ-PGA needs to be several tens to hundreds times lower than at present. With this vision in mind, a lot of work has been done with respect to the molecular biology aspects of γ-PGA production with different organisms. A complete understanding of γ-PGA synthesis has still not been achieved, especially with regard to the regulation of γ-PGA production within the cell. Statistical analyses of large-scale γ-PGA production could also lead to answers for cost-effective production of γ-PGA.

ω-PGA has been exploited widely for medical applications, more so for sustained drug delivery. Researchers have pointed out that there could be several advantages of using γ-PGA over ω-PGA. Pure γ-PGA can be obtained in large quantities without any chemical modification step. It is not susceptible to proteases and hence could provide better sustained delivery of conjugated drugs in the body. Better incorporation of drug in terms of quantity of drug conjugated into γ-PGA has been achieved. Optimization of γ-PGA with respect to molecular mass, enantiomeric composition and conformational states is an indispensable step in making application of γ-PGA practical. Molecular biology techniques would not only help optimize γ-PGA production for various applications, but also enable us to understand the mechanisms by which γ-PGA is effective in numerous applications. We hope this review provides an updated overall understanding of the concepts involved in γ-PGA synthesis and applications, and facilitates further research on this highly useful multifunctional biomaterial.

References


Edited by: S. Spiro