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Kevin M. Turner, Gianfranco Pasut, Francesco M. Veronese, Angela Boyce, Gary Walsh. Stabilization of a supplemental digestive enzyme by post-translational engineering using chemically-activated polyethylene glycol. *Biotechnology Letters*, 2010, 33 (3), pp.617-621. <10.1007/s10529-010-0474-7>. <hal-00642942>

HAL Id: hal-00642942

<https://hal.science/hal-00642942v1>

Submitted on 20 Nov 2011

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Section: Microbial and Enzyme Technology

Stabilization of a supplemental digestive enzyme by post-translational engineering using chemically-activated polyethylene glycol

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Abstract

Many enzymes used as digestive aids exhibit, at best, moderate stability when incubated under gastrointestinal conditions. A supplemental β -galactosidase administered orally to treat lactose intolerance was conjugated to 40 kDa, branched polyethylene glycol (PEG). PEGylation increased the enzyme's relative activity at lower pH values (2.5-4.5) and doubled enzyme stability at pH 2.5. The PEGylated enzyme retained significantly more residual activity after exposure to simulated gastric conditions (52% versus 31%), a consequence of protection from both pepsin and low pH mediated inactivation. Conjugation also provided significant protection against the proteolytic component of pancreatin. Overall, the PEGylated enzyme retained over twice the levels of residual activity recorded for non-PEGylated enzyme after exposure to complete simulated gastrointestinal conditions. PEGylation also marginally improved the enzyme's kinetic characteristics. When using its physiological substrate (lactose), K_m values recorded were slightly decreased (from 83 to 60 μM) and k_{cat}/K_m values ($\text{M}^{-1} \text{s}^{-1}$) were increased from 100 to 147. This appears to be the first report of the use of a conjugated PEG to stabilize a digestive enzyme and the first report of the ability of conjugated PEG to stabilize a protein at low pH.

Key words:

β -galactosidase; gastrointestinal; lactase; lactose intolerance; PEGylation

Introduction

A wide range of enzymes find application as oral digestive supplements, both for human and animal use (Bedford and Partridge 2001, O'Connell 2006). In order to be effective, digestive supplemental enzymes must be stable and active within the gastrointestinal tract, where they are exposed to various potentially inactivating influences such as low stomach pH and digestive proteases (Xenos et al. 1998). β -Galactosidase (lactase) is used to treat lactose maldigestion/intolerance, a condition affecting up to 75% of the world's population (Swagerty et al. 2002). Previous studies in the host laboratory indicate that supplemental lactases display poor stability when subjected to *in vitro* simulated gastrointestinal conditions, which may explain the often variable and sometimes disappointing clinical results obtained with these preparations (O'Connell and Walsh 2006). More recently, we (O'Connell and Walsh 2007, 2008) and others (Wang et al. 2009) have identified several previously uncharacterized β -galactosidases likely more suited to clinical application than current commercial preparations. However, even these enzymes are somewhat susceptible to digestive proteases and/or low pH values. Furthermore, their commercialization would be dependent upon further significant technical and regulatory investigation. *In vivo* stabilization of existing commercialized β -galactosidases represents an alternative and likely more attractive route to enhancing efficacy. The covalent attachment of polyethylene glycol (PEG) to therapeutic proteins destined for parenteral administration can increase their efficacy, largely by slowing the rate of glomerular filtration and/or decreasing immunogenicity (Pasut and Veronese 2007). PEGylation may also potentially protect therapeutic proteins from serum proteases via steric hindrance (Veronese et al. 1997). We theorize that PEGylation of supplemental β -galactosidases destined for oral administration might render them more stable by sterically preventing their proteolytic degradation within the digestive tract. This study aims to assess this possibility.

Materials and Methods

Materials

Commercial lactase digestive supplement (Trade name 'Lacteeze', derived from *Aspergillus oryzae*) was obtained from Gelda Scientific, Ontario, Canada. Chemically-activated, 40 kDa branched-PEG (mPEG2-NHS) was purchased from Nektar Therapeutics, Alabama, USA. Additional reagents were purchased from Sigma-Aldrich.

Methods

Assay of β -galactosidase was as described by O'Connell and Walsh (2006). Enzyme-PEG conjugation involved co-incubation of enzyme (10 mg/ml in 0.1 M borate buffer, pH 8.0) with mPEG2-NHS (1:10 molar ratio, 25°C with stirring, 4 h). Monitoring of conjugate formation and separation of conjugate from both unconjugated enzyme and free PEG was achieved using a Superdex 200 prep-grade, size-exclusion column (320 ml) attached to a HPLC system using 0.1 M sodium phosphate buffer pH 6.8 as mobile phase and 1 ml/min. SDS-PAGE analysis was undertaken using an 8% (v/v) gel with staining for PEG in accordance with (Kurfurst 1992). Kinetic properties and the effect of temperature and pH upon enzyme activity and stability was determined as described by O'Connell and Walsh (2007) while the effect of simulated gastrointestinal tract conditions were determined as described by O'Connell and Walsh (2006).

Results and discussion

Size-exclusion chromatography (Fig. 1) indicated successful PEGylation. The appearance of a single new peak of reduced retention time indicates formation of a monoPEGylated species (the new peak appearing at a longer retention time, 36 min, represents the NHS released from the coupling reaction, (Veronese et al. 2001). Percentage PEGylation was calculated at 55%, [well within the normal range typically achieved (Veronese 2001)]. MonoPEGylation was confirmed via a TNBS assay (Veronese 2001) and was also supported by SDS-PAGE analysis (Supplementary Fig. 1). In the context of Supplementary Fig. 1, PEG significantly distorts and broadens electrophoretic bands whilst PEG conjugation reduces SDS PAGE electrophoretic mobility, rendering it unsuitable for the molecular mass determination of conjugates (Zheng et al. 2007).

mPEG2-NHS targets accessible surface lysines. Although the primary or higher order structure of the commercial *Aspergillus oryzae*-derived lactase supplemental enzyme is unavailable, β -galactosidase sequences amongst *Aspergilli* are highly conserved (Ryttersgaard et al. 2002). Modelling of the β -galactosidase from *A. oryzae* ATCC 42149 (accession number Q2UCZ1) using PyMOL software (www.pymol.org) reveals 16 putative surface lysines. Even when multiple surface lysines are available utilization of high molecular weight, branched PEG generally results in mono-PEGylation, likely due to steric hindrance of further PEG attachment (Veronese 2001).

Collection of appropriate chromatographic fractions facilitated purification of the PEGylated species (Supplementary Fig. 1), allowing its application-relevant characterization. PEGylation of the β -galactosidase had no effect upon relative enzyme activity at varying temperatures or upon temperature stability but increased the enzyme's relative activity at lower pH values (pH 2.5 – 4.5; Fig. 2). PEGylation also doubled the enzyme's stability at low pH with the PEGylated enzyme retaining 22% (n = 3) residual activity after incubation at pH 2.5 for 30 min, whereas the unPEGylated enzyme retained only 11% (n=3) after similar treatment. In an application-relevant context, enhanced activity and stability at low pH is likely beneficial as it infers that the PEGylated enzyme would likely display significantly increased activity and stability at gastric pH (typically 2.0-5.5).

Independent exposure of both PEGylated and unPEGylated enzyme to simulated gastric and simulated small intestinal tract conditions indicated a clear PEG-stabilizing effect (Table 1). Incubation under simulated gastric conditions illustrates that: (a) enzyme inactivation is mediated by a combination of low pH inactivation, and proteolytic degradation by pepsin; and (b) that PEGylation provides protection from both influences. While possible protection from proteolysis was anticipated, and indeed formed the initial basis of this study, the stabilizing influence of conjugated PEG against low pH-based enzyme inactivation, to our knowledge, has not been reported previously. Despite consideration and extensively reviewing the pertinent literature no mechanism is obvious by which this is achieved. PEGylation also provides an overall stabilizing effect when incubated under simulated small intestinal conditions, though in this case the primary mode of stabilization is via protection against the proteolytic component of pancreatin (Table 1). After incubation under simulated total gastrointestinal conditions (SGF followed by SIF; Table 1), the PEGylated enzyme retained 25% of initial activity while the unPEGylated form under the same conditions retains only 12% of the initial activity.

PEGylation could potentially negatively affect enzyme kinetics; for example, if conjugation occurred at or near the enzymatic active site. This was a source of concern given that PyMOL-based modelling of the *A. oryzae* ATCC 42149-derived β -galactosidase revealed four putative lysines at its active site. However, PEGylation actually marginally enhanced the commercial enzyme's kinetic characteristics when lactose was used as a substrate (Table 2). PEGylation decreased the K_m value slightly (from 83 to 60 μM) and marginally increased the V_{max} value (8×10^{-3} v 9×10^{-3} $\mu\text{M min}^{-1}$), increasing the overall k_{cat} value (s^{-1}) from 8×10^{-3} to 9×10^{-3} , and increasing the k_{cat}/K_m value ($\text{M}^{-1} \text{s}^{-1}$) from 100 to 147. This data infers that the PEG is likely conjugated at a point distant from the active site and that it does not exert a detrimental effect upon enzyme conformation. The high molecular weight and branched (*i.e.* sterically bulky) PEG used was chosen specifically to minimize the possibility of attachment to active site lysines, given that the active site itself likely forms a somewhat less accessible crevice on the enzyme's surface.

In conclusion the results presented indicate that PEGylation improves the application-relevant characteristics of a supplemental commercial β -galactosidase used in the treatment of lactose intolerance and suggests a wider potential strategy for potential stabilization of supplemental digestive enzymes.

Acknowledgements

Work funded in part by the Irish Research Council for Science, Engineering and Technology.

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Figure captions:

Fig. 1

Overlay comparison of size exclusion chromatographic elution profiles of PEGylated and unPEGylated β -galactosidase. Profile 1, unPEGylated enzyme; profile 2 post-PEGylation profile

Fig. 2

pH versus activity profiles of PEGylated and unPEGylated enzyme. Data expressed as a percentage of maximum activity. 100% activity of the PEGylated enzyme equated to 1014 IU/ml, while for the unpegylated enzyme it equated to 1006 IU/ml activity. Each value represents mean \pm SD (n=3).

Table 1

The effect of *in vitro* simulated gastrointestinal conditions upon PEGylated and unPEGylated forms of supplemental β -galactosidase. Each value represents mean (n = 3). For each mean value, the associated SDs recorded were in all cases < 7% of that mean value

Treatment	% Residual Activity	
	UnPEGylated	PEGylated
SGF ^a	31	52
SGF-NPEP ^b	44	56
SIF ^c	7	32
SIF-NPAN ^d	48	60
SGF+ SIF	12	25

*Values expressed as a percentage of original activity recorded. 100% activity of the PEGylated enzyme equated to 1014 IU/mL, while for the unpegylated enzyme it equated to 1006 IU/mL activity

^asimulated gastric fluid (SGF), ^bsimulated gastric fluid without pepsin (SGF-NPEP), ^csimulated intestinal fluid (SIF),

^dsimulated intestinal fluid without pancreatin (SIF-NPAN).

Table 2

The effect of PEGylation upon selected kinetic parameters of the supplemental β -galactosidase enzyme using physiological substrate (lactose) as well as other commonly employed substrates (ONPG and PNPG)

	K_m (μM)	V_{max} ($\mu\text{M min}^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
Lactose				
'Lacteeze'	83	0.008	0.008	100
PEG-'Lacteeze'	60	0.009	0.009	147
ONPG				
'Lacteeze'	5	0.21	50	1×10^7
PEG-'Lacteeze'	5	0.20	134	3×10^7
PNPG				
'Lacteeze'	0.05	5	1083	2×10^{10}
PEG-'Lacteeze'	0.06	4	2493	4×10^{10}

*Kinetic parameters were calculated using the synthetic substrates ONPG/PNPG as well as the natural substrate lactose as described by O'Connell and Walsh (2007). Where appropriate, values are expressed as whole numbers. Each value represents mean ($n = 4$). For each mean value, the associated SDs recorded were in all cases $< 1\%$ of that mean value.

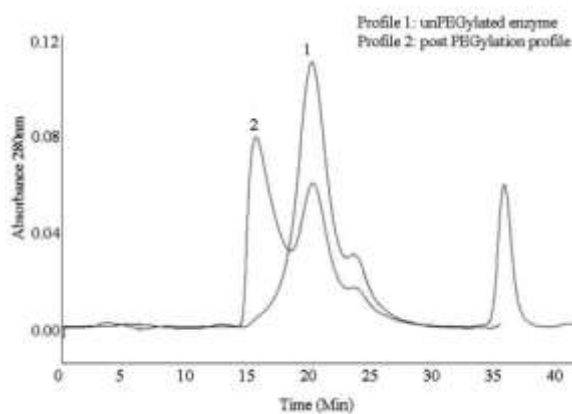


Fig. 1

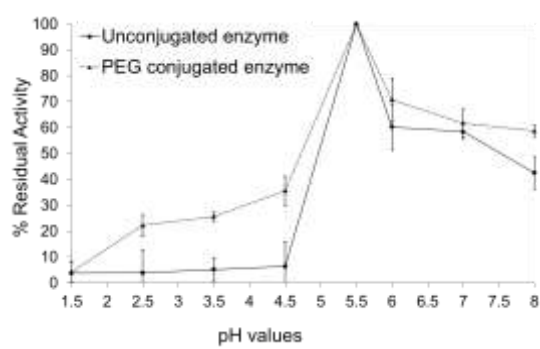


Fig. 2