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Enzyme glycation influences product yields during oligosaccharide synthesis by reverse hydrolysis

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Abstract

Possible evidence is presented for Maillard glycation of enzymes during oligosaccharide synthesis by reverse hydrolysis. In 70% (w/v) mannose solutions, 1,2-α-mannosidase from *Penicillium citrinum* lost 40% and α-mannosidase from almonds lost 60% activity at 55 °C over 2 weeks. Oligosaccharide yields were 15 and 45% respectively. Higher molecular weight glycation adducts were formed in a time-dependent manner as seen by MALDI-TOF. Inhibitors of the Maillard reaction were able to partially alleviate these effects resulting in reduced loss of enzyme activity and oligosaccharide yield increases of 27–53% relative to the control.

Keywords: Maillard reaction; α-Mannosidase; Enzymatic synthesis; Oligosaccharide synthesis; Reverse hydrolysis

1. Introduction

Enzymatic synthesis reactions are an attractive means of producing biologically active oligosaccharides due to their simplicity and regioselectivity [1]. Of the available approaches to the enzymatic synthesis of oligosaccharides, the use of glycosidases in reverse has several advantages. Glycosidases are readily available from a range of plant and microbial sources, reverse hydrolysis or condensation reactions are simple to perform [2] and if the correct enzyme is chosen they can have absolute selectivity [3]. The biggest disadvantage is that the product yields tend to be rather low. Overall process yields, however, can be increased by recycling the unused substrate, which is not destroyed in the reaction. Despite this, it would be desirable to maximize the reaction yields. Examination of the literature in this area reveals a range of product yields for such reactions, from 14 [4] to 70% [5]. This wide variation in the yields suggests a possible role of enzyme inactivation in addition to thermodynamic limitation of the reaction, as reaction conditions used are generally similar (50–80% total sugar (w/w), 50–60 °C). A likely mechanism of enzyme inactivation under the conditions of the reaction could be Maillard glycation of the enzyme. The Maillard reaction has been postulated as a limiting factor in oligosaccharide synthesis reactions [6–9] but little evidence for this has been reported to date. In this study, we were able to show that Maillard induced inactivation of glycosidase enzymes occurs during oligosaccharide synthesis by reverse hydrolysis. We investigated the use of a few known inhibitors [10] of the Maillard reaction to increase the yields of oligosaccharides in condensation synthesis reactions catalysed by α-1,2-mannosidase from *Penicillium citrinum* and α-mannosidase from almonds.

2. Materials and methods

2.1. Fungal strain

Aspergillus oryzae strain PM-1 overproducing the 1,2-α-mannosidase of *P. citrinum* was kindly provided by Dr. Takashi Yoshida of Hirosaki University, Japan. It was maintained on agar slopes containing 2% malt extract, 0.2% bactopeptone and 2% glucose at 4 °C.

2.2. Enzymes

For *A. oryzae* recombinant α-mannosidase production, spores of PM-1 were inoculated into 1 l of DPY medium

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[11] at 1×10^5/ml and incubated at 30 °C on an orbital shaker at 150 rpm for 3 days. The mycelia were separated from the medium by filtration through Miracloth (CN Biosciences, Nottingham, UK). The filtrate was passed through a 0.2 μm filter and then concentrated by ultrafiltration through a 10,000 nominal molecular weight cut-off (NMWCO) ultrafiltration membrane (Millipore, Watford, UK) using a Gyrosep™ 300 ml stirred cell from Intersep (Wokingham, UK). The pH of the enzyme preparation was adjusted to 5.0 by diafiltration with 0.1 M sodium acetate buffer of the same pH. Further concentration of enzyme was achieved using Vivaspin centrifugal concentrators (Vivascience, Epsom, UK) with 10,000 NMWCO and this was used as the enzyme preparation for synthesis reactions.

Almond α-mannosidase was obtained from Sigma (Dorset, UK) and used as such.

2.3. Reagents

All reagents and inhibitors of Maillard reaction were purchased from Sigma (Dorset, UK) unless otherwise specified. Maillard inhibitors used in the study were 2-aminoguanidine hydrochloride (2-AG), sodium metabisulfite (MBS), *o*-phenylene diamine dihydrochloride (OPD) and semicarbazide hydrochloride (SMC).

2.4. Equilibrium synthesis reactions

Synthesis reactions were set up at 70% (w/w) mannose in 0.1 M sodium acetate buffer at pH 5.0 for both *P. citrinum* α-mannosidase and almond α-mannosidase, with and without inhibitors of Maillard at a concentration of 10 mM. Enzyme was added at 0.5 and 2 U/ml of the reaction mixture and incubated at 55 °C for 2 weeks. In the case of the purified *P. citrinum* enzyme this corresponded to 6.6×10^{-5} M enzyme in the reaction mixture. The reactions were sampled for analysis every alternate day until maximum product yields were attained. Two samples were taken at each time point, one of which was boiled to inactivate the enzyme and used for HPLC analysis and the other frozen immediately without boiling for use in enzyme activity assays and electrophoresis.

2.5. Enzyme assays

The enzymes were recovered from the synthesis mixtures using Vivaspin centrifugal concentrators (Vivascience, Epsom, UK) with 10,000 NMWCO. The samples from the reaction mixtures were diluted to 20 ml with 0.1 M sodium acetate buffer, pH 5.0 and then concentrated down to 1 ml. Two more washes were repeated with the same buffer and the enzyme from each reaction sample was thus obtained in concentrated form, free from other reaction components.

A. oryzae recombinant α-mannosidase activity was determined using baker's yeast mannan as substrate and measuring the liberated mannose by Nelson Somogyi method [12]. One enzyme unit was defined as the amount of enzyme required to liberate 1 μmol of mannose from mannan per minute at 30 °C and pH 5.0.

Almond α-mannosidase was assayed by incubating suitably diluted enzyme in 0.1 M sodium acetate buffer, pH 4.5 with 5.5 mM *p*-nitrophenyl α-D-mannopyranoside at 30 °C for 10 min and determining the *p*-nitrophenol released by absorbance measurement at 420 nm, after terminating the reaction by addition of 0.1 M sodium carbonate. The unit of activity was the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol per min under the assay conditions.

Protein concentration was determined using the bicinchoninic acid (BCA) assay [13]. Residual enzyme activity was calculated as % specific activity (U/mg) with respect to the enzyme specific activity at the start of the synthesis reaction.

2.6. SDS–PAGE

The relative molecular mass of the purified enzyme was determined by analysis in 10% SDS–PAGE gels against wide-range SigmaMarker (Sigma, Poole, UK). The bands were visualized by Coomassie blue stain or by silver staining [14].

2.7. Purification of oligosaccharide products

Products of synthesis reactions were purified by gel-filtration on a BioGel P-2 (Bio-Rad, Waford, UK) column (960 mm × 60 mm), eluted with deionised water at a flow rate of 2 ml/min. Carbohydrates were detected using a Gilson 132 refractive index detector (Anachem, Luton, UK). Fractions containing mannobiose were pooled and lyophilized.

2.8. Oligosaccharide degradation

A 14% (w/v) solution of purified mannobiose synthesised using *P. citrinum* α-1,2-mannosidase was prepared in 0.1 M sodium acetate buffer at pH 5.0 and analysed by HPLC following incubation at 55 °C for 7 days.

2.9. Analysis of products by HPLC

HPLC was performed using a Merck Hitachi La Chrom system (VWR International, Poole, UK) with a L-7490 refractive index detector. Synthesis products were analysed on a 250 mm × 4.6 mm Apex carbohydrate 5 μ column (Jones Chromatography, Mid-Glamorgan, UK) in 75% (v/v) acetonitrile in water, eluted at 0.8 ml/min.

2.10. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)

The laser desorption/ionization experiments were performed on a BIFLEX III time-of-flight instrument (Bruker-Franzen Analytik, Bremen, Germany) operated in the positive mode. Samples were analysed in the linear mode, and typically 100 laser shots were summed into a single mass spectrum. External calibration was performed, using BSA as the standard. A saturated solution of sinapinic acid in acetonitrile:water (1:2) with 0.1% TFA was used as the matrix. The sample and the matrix solution (0.5 μl each) were spotted onto the target and dried at room temperature.

3. Results and discussion

3.1. Enzyme purity

The almond enzyme from Sigma was used without further purification. The *P. citrinum* enzyme was purified by ultrafiltration from cultures of recombinant *A. oryzae*. This process resulted in a predominant band on SDS–PAGE (Fig. 1) with low quantities of contaminating proteins. The molecular weight of the enzyme was determined as 53,000 Da by SDS–PAGE. This corresponded well with the value of 53,661 Da determined by MALDI-TOF (See below).

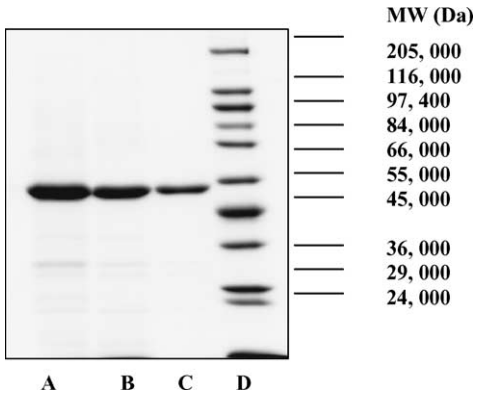

Fig. 1. SDS–PAGE gel of recombinant α-1,2-mannosidase from *P. citrinum* expressed in *A. oryzae*. Lane A, B, C: concentrated enzyme preparation; lane D: molecular weight marker (wide-range SigmaMarker). The molecular weight of each band of the protein marker is shown on the right.

3.2. Enzyme activity over the course of reverse hydrolysis reaction

The time course of oligosaccharide synthesis using both enzymes is presented in Fig. 2. The optimisation and products of these synthesis reactions have been described elsewhere [15]. *P. citrinum* α-1,2-mannosidase produces α-1 → 2-linked products, whereas the almond enzyme produces a mixture of α-1→6, α-1→3 and α-1→2-linked products. In order to correlate the product yields and the enzyme activity over the course of synthesis, the residual activity of *P. citrinum* α-mannosidase and almond α-mannosidase during the course of their respective synthesis reactions was determined and is shown in Fig. 3. *P. citrinum* enzyme lost about 40% and the almond enzyme 60% activity over the course of the reaction. Neither enzyme underwent complete inactivation. The rate of inactivation was higher in the first 6 days of reaction and stabilized as the reaction approached apparent equilibrium.

The effect of incubation of *P. citrinum* enzyme in the synthetic mixtures on its molecular structure was investigated by

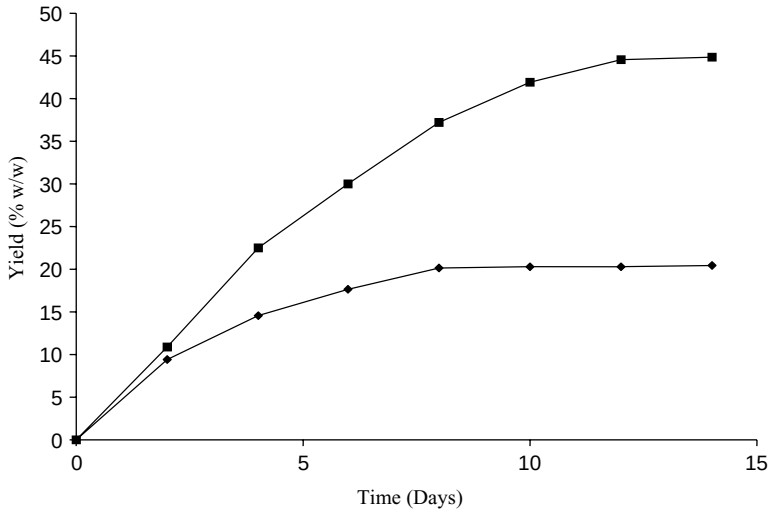

Fig. 2. Time course of oligosaccharide synthesis with α-1,2-mannosidase from *P. citrinum* (◆) and α-mannosidase from almond (■). Synthesis reactions were set up at 70% (w/w) mannose in 0.1 M sodium acetate buffer at pH 5.0. *P. citrinum* α-1,2-mannosidase or almond α-mannosidase was added at 0.5 or 2 U/ml of the reaction mixture respectively and incubated at 55 °C for 2 weeks. The reactions were sampled for analysis by HPLC on alternate days until maximum yields were attained.

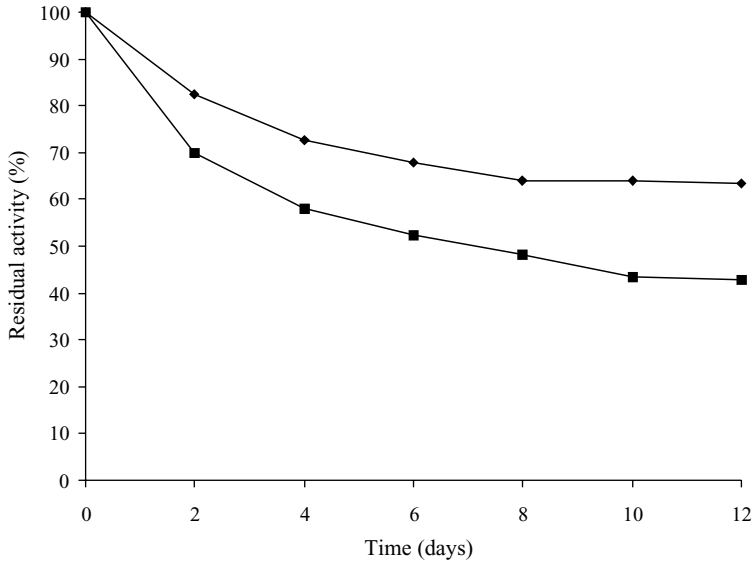

Fig. 3. Residual activity of *P. citrinum* α-1,2-mannosidase (◆) and almond α-mannosidase (■) over the course of oligosaccharide synthesis by reverse hydrolysis. Synthesis reactions were set up at 70% (w/w) mannose in 0.1 M sodium acetate buffer at pH 5.0. *P. citrinum* α-1,2-mannosidase or almond α-mannosidase was added at 0.5 or 2 U/ml of the reaction mixture respectively and incubated at 55 °C for 2 weeks. Samples were taken every 2 days and the residual enzyme activity determined after removal of carbohydrates.

MALDI-TOF (Fig. 4). MALDI-TOF mass spectra suggest a heterogeneous distribution of glycation products based on a significant mass increase and peak broadening.

Maillard reactions have been suspected by several authors of causing yield losses in enzymatic transglycosylation reactions [6–9]. Most of this interest has come from the use of extremophile enzymes in galacto-oligosaccharide synthesis. Such enzymes are extremely thermostable and this facilitates their use at high temperatures. The elevated temperatures in turn allow higher concentrations of lactose to be used in the reactions, increasing oligosaccharide yields [6]. Although reverse hydrolysis reactions generally do not require such high temperatures, they do require extremely high monosaccharide concentrations and this is likely to promote the Maillard reaction between the reducing monosaccharides and the arginine and lysine residues in the protein.

There are few reports on changes in enzyme activity and stability in reverse hydrolysis [5,16] correlating it with product yields. Enzyme activity for the most part is not considered a significant factor in these reactions as it is usually added in excess and is expected to be protected against thermal denaturation by the high substrate concentrations. Since thermal denaturation is likely to be low, we suspected whether Maillard glycation could be a contributing factor to the enzyme inactivation observed in this study. Varying degrees of browning have been observed in synthesis reactions using different enzymes and sugars in our laboratory. Increased browning was observed in reactions carried out in phosphate buffer at pH 6.0 compared to acetate buffer at pH 5.0. Phosphate is known to increase protein glycation [17]. Presence of cations also increased browning as observed with Zn^{2+}-containing buffer of Jack bean α-mannosidase-catalysed reverse hydrolysis reactions. These observations suggest that Maillard reaction could occur in reverse hydrolysis reactions. It is thus likely that the enzyme in the reaction would undergo glycation and consequently, possible inactivation.

Study of enzyme activity and molecular structure for the two enzymes used in this study provides suggestive evidence for glycation. Consideration of the mass change seen suggests that cross-linked dimers or oligomers were not formed. The extent of glycation may be regulated by several factors including the reaction pH, temperature, sugar type, stability, concentration, the amount of sugar in the open chain form and the protein [18–20]. Recently [21], by employing lysine-free peptides containing arginine or vice-versa, it has been shown that to form a cross-link, a free amino group is needed, either from a lysine residue or the N-terminus. The low water activity of the reaction might also influence Maillard reaction because of diffusional limitations [22]. MALDI-TOF MS has been used as both a qualitative and quantitative tool for analysing glycated proteins. The increase in molecular weight and peak broadening observed for the *P. citrinum* mannosidase are consistent with observations for glycated proteins both in model systems such as BSA and glucose, as well as in clinical samples such as from diabetic subjects [23,24].

Maillard glycation is known to cause enzyme inactivation due to structural and conformational changes [25–27]. Gradual inactivation of human erythrocyte biphosphoglycerate mutase was observed during in vitro glycation with reducing sugars glucose and fructose. This inactivation was due to modification of several lysine residues, particularly Lys-158 which is probably in the catalytic region of the

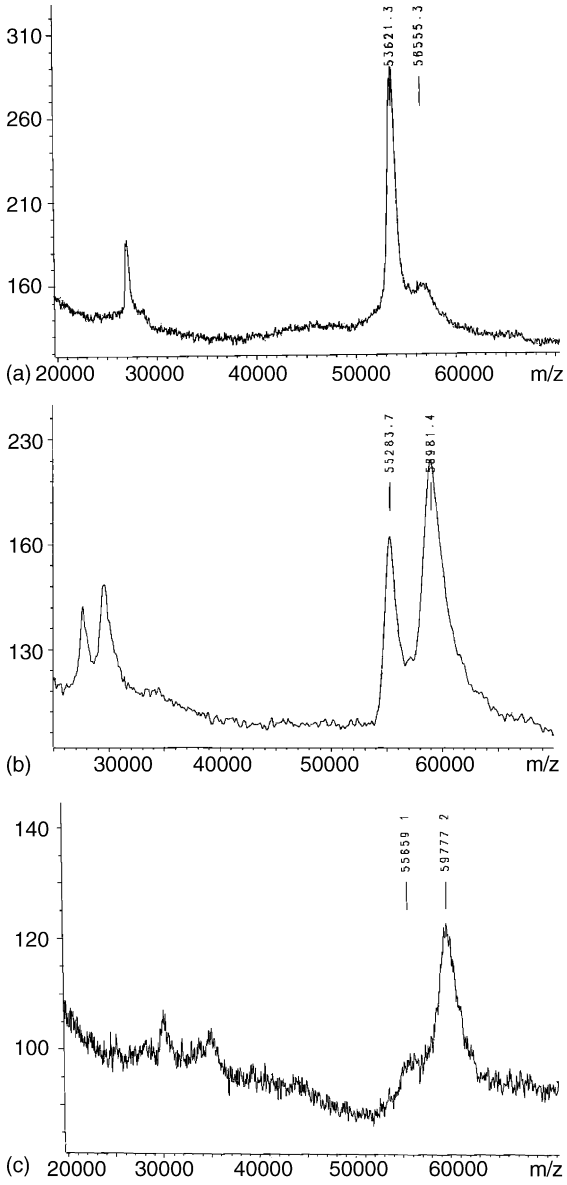

Fig. 4. MALDI mass spectra of *P. citrinum* α-1,2-mannosidase recovered from synthesis reactions at time points (a) 0 days (b) 2 days and (c) 6 days.

enzyme [26]. Inactivation of the enzyme by glycation also depends on the type of sugar in the reaction [25,26]. Only partial inactivation of human erythrocyte biphosphoglycerate mutase was observed with glucose whereas fructose led to complete inactivation. A similar trend was observed during inactivation of sorbitol dehydrogenase, where Lys-210, Lys-319 and Lys-369 have been suggested as possible glycation sites responsible for inactivation. An interesting result was obtained by Seidler and Seibel [27] wherein the activity of aspartate aminotransferase increased on brief exposure to glycating agents, presumably due to a change in conformation leading to increased domain mobility. Prolonged incubation with the glycating agents, however, led to decreased catalytic activity.

3.3. Effect of Maillard inhibitors

The effects of four known inhibitors of the Maillard reaction were determined for *P. citrinum* α-1,2-mannosidase (Fig. 5). The yield of the oligosaccharide in the absence of the inhibitors was 15%. Addition of the inhibitors resulted in yield increases of 27–53% relative to the control. Amongst the inhibitors used in the study, *o*-phenylene diamine dihydrochloride (OPD) showed highest improvement in product yields of 53% relative to the control and therefore was chosen for further study. The effect of OPD on the synthesis of oligosaccharides by *P. citrinum* α-1,2-mannosidase and almond α-mannosidase is shown in Fig. 6. Presence of inhibitor increased both rate of reaction and final product yield in case of *P. citrinum* enzyme. When the almond α-mannosidase was used, addition of OPD resulted in a faster rate of reaction although the final yields for the two reactions in the presence and absence of OPD appear to be converging. Incubation in the presence of Maillard inhibitors had no effect on product composition as determined by High Performance Anion Exchange Chromatography (data not shown).

The enzyme activity over the course of the reaction (Fig. 7) was also investigated in the presence of OPD. In the presence of inhibitor, the rate of inactivation was slower; the *P. citrinum* enzyme lost little activity up to day 4 of reaction. Almond enzyme lost activity from the onset of the reaction even in presence of the inhibitor. However, the extent of inactivation in the presence of inhibitor was lower. The formation of the glycated species was delayed when *P. citrinum* reactions were carried out after incorporating OPD (Fig. 8). The total increase in molecular weight as detected by MALDI-TOF was 7037 Da, corresponding to the condensation of 44 mannose units. In reactions containing OPD, the increase in molecular weight was 6367 Da.

OPD, 2-AG and its analogue SMC act by trapping the dicarbonyl-intermediates [10] such as glyoxal, methylglyoxal and deoxyglycosones [28] formed during the early stages of Maillard reaction. MBS acts by sulfating or blocking the reactive hydroxyl groups of Maillard reaction intermediates. The inhibitors thus prevent the formation of advanced-glycation end products (AGEs). These inhibitors have been reported in literature for their inhibitory potential for AGEs implicated in diseases such as diabetes and cataract [10,29]. The dicarbonyls can be formed via various routes, either from the Amadori product, or by the degradation of the sugar [30,31]. The oligosaccharides formed are themselves capable of undergoing hydrothermolysis and isomerizations under aqueous conditions via a “peeling off” mechanism, to form degradation products capable of serving as starting materials in further Maillard reaction pathways [32]. Low pH and sodium salts can also increase oligosaccharide degradation [33]. Reaction of free glycosidic OH group can initialize breakdown of the sugar molecule by uncatalysed thermal degradation. This was observed when a solution of

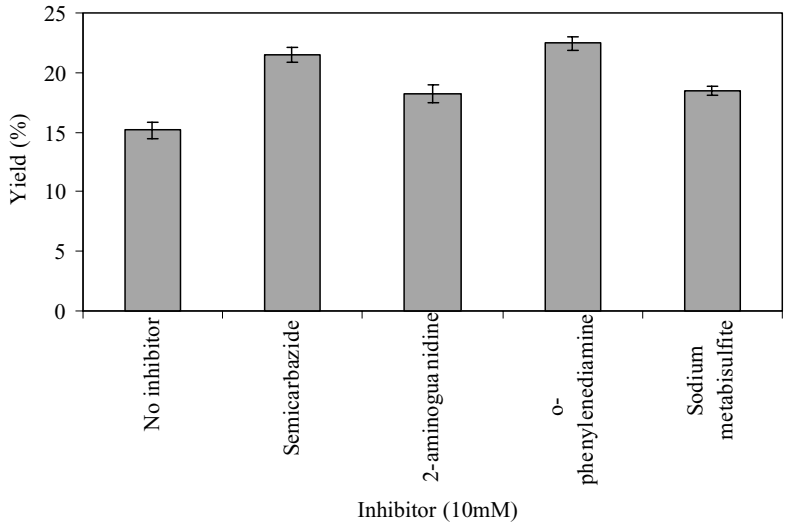

Fig. 5. Yields of Manα-1–2Man in the presence of various inhibitors of the Maillard reaction. Synthesis reactions were set up at 70% (w/w) mannose in 0.1 M sodium acetate buffer at pH 5.0. *P. citrinum* α-1,2-mannosidase was added at 0.5 U/ml of the reaction mixture and incubated at 55 °C for 2 weeks. Maillard inhibitors were incorporated at 10 mM. The reactions were sampled for analysis by HPLC on alternate days until maximum yields were attained. Results presented are the mean ± S.D. of three different determinations.

disaccharide synthesised using the *P. citrinum* enzyme was incubated at the reaction temperature of 55 °C for a week and this could not be inhibited by the presence of OPD (data not shown). The partial degradation of oligosaccharide observed could further limit final product yields. Since the inhibitors used in the study function by a common mechanism, they might not be able to prevent Maillard reaction occurring by other routes, which might account for incomplete inhibition.

Maximum oligosaccharide synthesis occurred within the first 2–4 days of the reaction (Figs. 3 and 5), before the enzyme has undergone appreciable inactivation. In reactions with inhibitor, higher yields could be accumulated because of reduced enzyme inactivation. In case of almond enzyme, synthesis proceeds almost linearly, doubling at every time point until reaching a maximum value. Higher rates of product formation were obtained in presence of the inhibitor, but final yields were not appreciably increased.

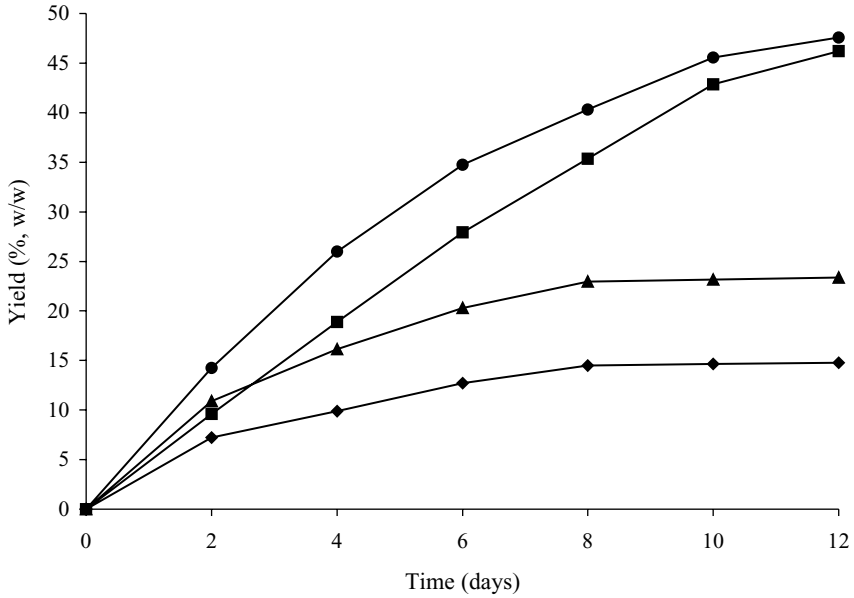

Fig. 6. Effect of OPD on synthesis of oligosaccharides. Effect of OPD on synthesis of oligosaccharides with almond α-mannosidase in the presence (●) and absence (■) of OPD, and with *P. citrinum* α-1,2-mannosidase in the presence (▲) and absence (◆) of OPD. Synthesis reactions were set up at 70% (w/w) mannose in 0.1 M sodium acetate buffer at pH 5.0. Almond α-mannosidase or *P. citrinum* α-1,2-mannosidase was added at 0.5 or 2 U/ml of the reaction mixture respectively and incubated at 55 °C for 2 weeks. The reactions were sampled for analysis by HPLC on alternate days until maximum yields were attained.

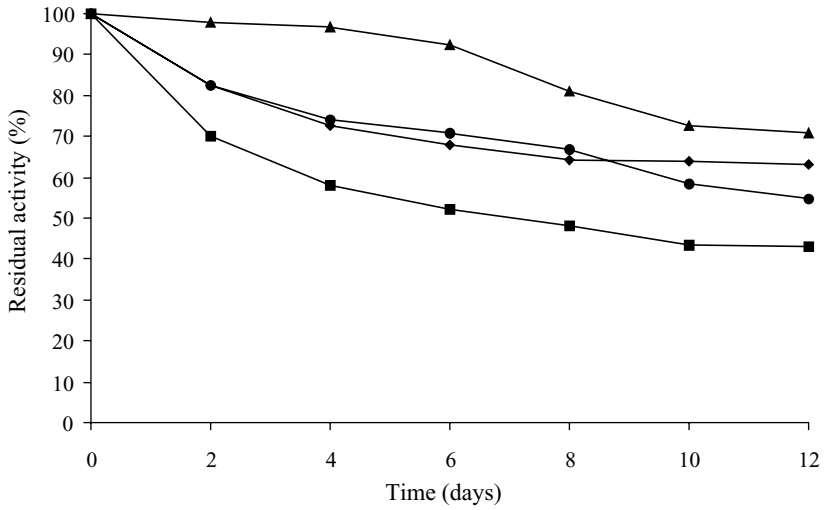

Fig. 7. Residual activity during the enzyme reactions. Residual activity of almond α-mannosidase in the presence (●) and absence (■) of 10 mM OPD, and with *P. citrinum* α-1,2-mannosidase in the presence (▲) and absence (◆) of 10 mM OPD. Synthesis reactions were set up at 70% (w/w) mannose in 0.1 M sodium acetate buffer at pH 5.0. Almond α-mannosidase or *P. citrinum* α-1,2-mannosidase was added at 0.5 or 2 U/ml of the reaction mixture respectively and incubated at 55 °C for 2 weeks. Samples were taken every two days and the residual enzyme activity determined after removal of carbohydrates.

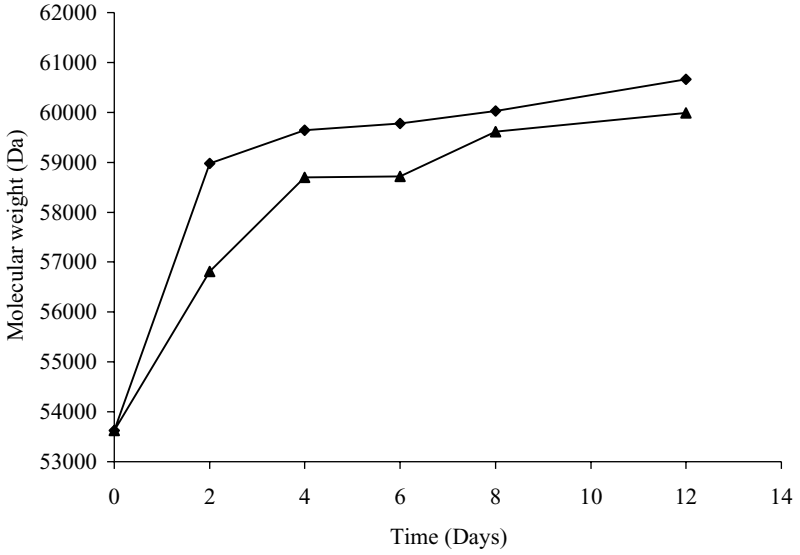

Fig. 8. Increase in molecular weight (Da) of *P. citrinum* α-1,2-mannosidase as a function of incubation time (days) of enzyme from reactions carried out in the presence (▲) and absence (◆) of OPD.

4. Conclusions

The study provides suggestive evidence for the role of Maillard glycation and enzyme inactivation as a limiting factor in attainment of equilibrium in oligosaccharide synthesis by reverse hydrolysis using mannosidases. Final yields of oligosaccharides could be increased to some extent by the incorporation of Maillard reaction inhibitors into the reaction medium. One inhibitor, OPD, was chosen for further study as it proved to be most effective in the initial screen. OPD protected both enzymes from inactivation during the synthesis reactions.

Control of Maillard reaction during oligosaccharide synthesis has been investigated. Bruins et al. [9] attempted enzyme immobilization as a means of reducing Maillard reaction effects during oligosaccharide synthesis using hyperthermophilic β-glycosidase from *Pyrococcus furiosus*; however, immobilization was unsuccessful in controlling Maillard reactivity. Adaptation of process conditions, chemical modification or biochemical engineering of the enzyme were suggested as possible solutions. The process conditions in this study have been adapted for controlling Maillard by incorporating inhibitors of the Maillard reaction in the synthesis mixtures.

The structure of the *P. citrinum* enzyme has been solved [34] and it contains 16 arginine and 28 lysine residues [35]. A high number of these residues can be expected to increase the propensity of the enzyme for glycation. Two of the arginine residues (Arg 126 and Arg 407) are in the active site of the enzyme and many of the lysine residues are conserved between the *P. citrinum*, human and yeast α-1,2-mannosidases, suggesting that they have an important function in the folding of the protein. Arg 407 is in a sequence region with several basic amino acids, and this might render it a possible site of glycation. Intense browning was not observed during oligosaccharide these synthesis reactions as would be expected with lysine, further suggesting that arginine might be the amino-acid undergoing glycation as it belongs to the low-browning producing group of amino-acids [36].

A disadvantage of some inhibitors used in the study (OPD, 2-AG and SMC) is that they are toxic compounds that might limit their use in food applications. Use of food-grade inhibitors such as MBS or deglycating enzymes (Amadoriases) [37,38] could be explored in such cases.

In conclusion, Maillard glycation of 1,2-α-mannosidase from *P. citrinum* and α-mannosidase from almond appears to be a factor influencing product yields of oligosaccharides synthesised using these enzymes. Use of Maillard inhibitors might provide a convenient way of improving the typically low oligosaccharide yields obtained in reverse hydrolysis and enable the enzymes to achieve their synthetic potential. The degrees of yield improvement seen in this study were low, but could conceivably be improved by optimisation of the Maillard inhibition strategy used.

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