Biocompatible functionalisation of starch

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1. Full Experimental Details

1.1 Reagents

10-Undecynoic acid, potato amylose, lipase from *Thermomyces lanuginosus* (lipolase, L0777), subtilisin (protease from *Bacillus licheniformis*, Type VIII, lyophilised powder), sodium ascorbate and copper sulphate were purchased from Sigma (UK). Tapioca, Amioca and Hylon VII starches were gifts from National Starch (Germany), wheat and maize starches were gifts from BASF (Germany). Alexa Fluor 488 azide, PEG4 carboxamide-6-azidohexanyl biotin (biotin azide) and streptavidin-Alexa Fluor 488 conjugate were purchased from Invitrogen (Molecular Probes, UK).

1.2 Enzymatic synthesis of starch 10-undecynoate (lipase in concentrated aqueous system)

A suitable (depending on the required concentration of the gelatinised starch solution) amount of sodium phosphate buffer (pH=7.0, 100 mM) is mixed with 2 g of starch (dried overnight in an oven at 50^{o}C), in a 50 cm^{3} round-bottom flask. The mixture is incubated in a water bath, for 1 hr at approximately 90^{o}C, while mechanically mixed with a Heidolph overhead stirrer (anchor paddle, 50rpm), thus gelatinising the starch, and then

cooled down to 50°C. Next 0.5 cm^3 of lipase (50kU, Sigma assay) and 0.55 g of 10-undecynoic acid (0.46 mol per mol anhydroglucose), per g of starch, are added to the gelatinised starch and the reaction is mixed while incubated at 50°C. After reaction completion the starch is precipitated from the reaction mixture by tenfold dilution with acetone and then washed twice with equal volumes of acetone. Finally it is dried in a vacuum desiccator for 1 hr and left overnight at a low temperature (40-50°C) in a convection oven. Alternatively some reactions were run on smaller scale (100mg of starch), in 2 cm^3 screw-top micro-centrifuge tubes. These are gelatinised in a heating block and the reaction is incubated in an air-incubator (Stuart SD60), while mixed with the aid of a Vortex Disruptor and the addition of glass beads.

1.3 Transesterification/GC analysis of starch esters

A small sample (5-30 mg) of esterified starch dissolved in 0.5 cm^3 DMSO was mixed with 1 cm^3 of sodium methoxide 0.07 M in methanol solution and a known amount (0.01 cm^3 of a 100 mg cm^{-3} solution in n-heptane) of internal standard (n-tridecane was suitable in the case of 10-undecynoic acid). Reagents were of anhydrous grade to minimise hydrolysis of the methyl ester as a side-reaction. This mixture was then heated (50°C) under reflux for 60 min, while shaken, then cooled and 1 cm^3 of deionised water and 1 cm^3 of n-heptane were added. The mixture was shaken for 1 min and left to settle. The top organic phase contained the methyl ester and could be removed and injected into the GC-FID (Perkin-Elmer Autosystem XL with a CP Simdist capillary column, oven set at 120°C, the injector at 130°C and the detector at 150°C).

1.4 Subtilisin catalysed synthesis of starch-6-hexynoate in isooctane (solubilised enzyme in organic solvent system)

Subtilisin solubilised in 2mM isooctane (1mg cm^{-3}) is prepared according to literature[1]. Dry starch is suspended in the isooctane/subtilisin solution (20 mg cm^{-3}, pure isooctane/AOT for non-enzymatic control) and methyl-5-hexynoate (threefold molar excess over hydroxyl groups in starch) is added. The mixture is incubated at 37°C, mixed by inversion for 48 hr, after which time, the starch is centrifuged and the isooctane removed. The polysaccharide is further washed in excess isooctane and twice in diisopropyl ether, in order to remove unreacted methyl-5-hexynoate.

1.5 Conjugation of fluorophore

In a 2 cm^3 glass vial, 2 mg (0.012 mmol) of starch sample are suspended in 0.4 cm^3 of sodium phosphate buffer (20mM pH=7.0), containing 1.0 mg (0.005 mmol) sodium ascorbate, 0.4 mg (0.0025 mmol) copper sulphate and 2.5 μg (0.003 μmol) Alexa Fluor 488 azide. The suspension is mixed in the dark, on a rotating mixer for approximately 30 min. After this time the suspension is diluted to three times its initial volume with deionised water and moved to 2 cm^3 disposable microcentrifuge tubes. Next it is centrifuged (Eppendorf 5415D, 10000rpm for 1 min) and the supernatant discarded. The starch is further washed five times with 1.5 cm^3 deionised water, to remove any residual unreacted fluorescent azide.

1.6 Biotinylation of starch and streptavidin attachment

Under the same conditions as described above (section 2.4), where the Alexa Fluor azide was replaced by biotin azide, a biotinylated starch was prepared. This sample was then suspended (1 mg product in 0.5 cm^3) in phosphate buffered saline (PBS, pH=7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4) and mixed with a Streptavidin-Alexa Fluor 488 conjugate (for 1 cm^3 product suspension, 0.05 cm^3

conjugate solution in PBS which contains 2mg of Invitrogen conjugate preparation per cm^3) for approximately 10 min. The streptavidin bound to the derivatised starch was visualised by fluorescence microscopy. The sample was washed 3 times with 1 cm^3 PBS to remove any non-covalently adsorbed streptavidin.

1.7 Fluorescence microscopy

The starch sample was suspended in deionised water (2 mg in 1 cm^3) and a drop was mounted onto a glass slide. The samples were viewed on a Zeiss Axio Imager A1 (x40 A-plan Zeiss), with an HBO 50 UV source. Carl Zeiss Filter set 09 ($\lambda_{excitation}$=450-490 nm, $\lambda_{emission}$>515 nm) was employed, where the presence of Alexa Fluor azide is detectable due to strong green fluorescence. The photomicrographs were taken with a Cannon Powershot G9 digital camera, attached to the microscope with a 25mm lens adaptor.

1.8 Fluorimetry

The starch sample was dissolved in DMSO (1 mg cm^{-3}) and the solution was placed in a UV grade cuvette. The sample was excited at 495 nm (the excitation maximum for Alexa Fluor 488) and the emission spectrum was recorded in the range of 500-600 nm, where a clear maximum could be observed at 520nm for fluorescent samples. Measurements were performed on a Jasco FP-6500 Spectrofluorometer (concave holographic grating excitation and emission monochromators).

2. Further Results

2.1 Enzymatic synthesis of starch 10-undecynoate

The lipase catalysed esterification of starch and 10-undecynoic acid was investigated for a number of different starch types (Figure 1a). It was observed that increased DS values were obtained for starches with higher amylopectin fractions, in accordance with our

previous studies with decanoic acid (Alissandratos et al-submitted). Amioca (99% amylopectin) and tapioca (83%) showed higher substitution than wheat, maize (both 75%), Hylon VII(30%) and pure amylose (ratios as given by producers). The reaction progress was monitored and a plateau of reactivity was observed after approximately 60 min (Figure 1b).

The reaction behaviour was also investigated as a function of the concentration of water in the starch gelatinisation mixture, prior to the enzymatic reaction. This was previously found to have a major effect on the reaction with decanoic acid. The more water used in this mixture, the more water will be present in the enzymatic mixture as part of the "polysaccharide phase". From the graph (Figure 1c) it is apparent that there is a sharp optimum DS for 1 mL water per g starch. In agreement with our previous reports, the enzymatic synthesis is thermodynamically favoured by lower water concentrations, however there is a minimum amount of water required for the initial gelatinisation step to proceed resulting in an overall optimum of water content for the reaction.

The initial concentration of fatty acid was also found to influence the system (Figure 1d). Note that in all cases the fatty acid is in large excess, so any changes in conversion may be explained by changes in the available interfacial area. For lower concentrations of fatty acid the interface may be smaller leading to a lower DS. For very high initial concentrations of fatty acid the conversion observed after one hour decreases. It is noteworthy that at these concentrations the fatty acid was visible as a distinct phase, this may also suggest decreased dispersion in the gelatinised starch.

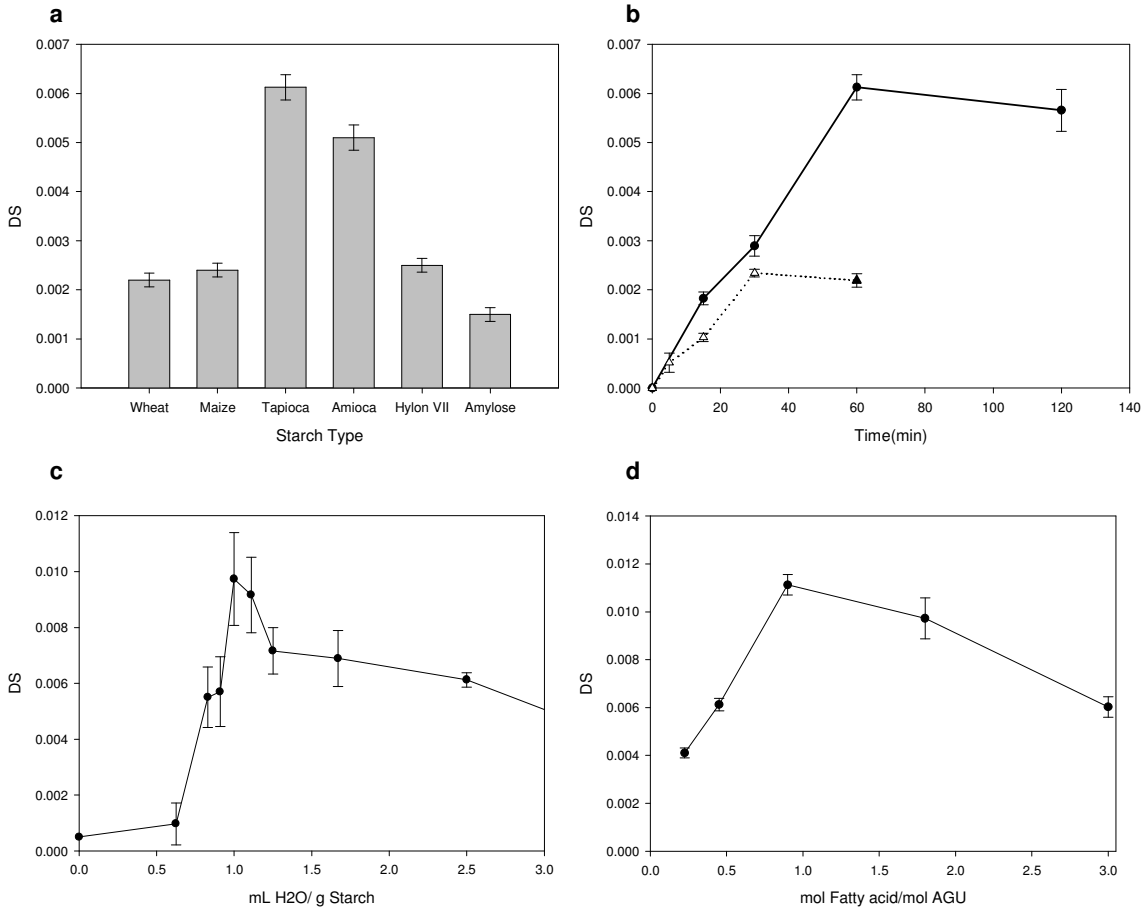

Figure 1. Lipase catalysed esterification of starch and 10-undecynoic acid (60 ^{o}C), a) for different starch types (60 min), b) reaction progress for tapioca (●) and wheat (Δ) starch, c) for varying concentration of water in initial gelatinisation mixture (tapioca, 60 min), d) for varying initial concentration of fatty acid (tapioca, 60 min).

2.2 Click derivatisation of starch ester with time

Within the click-system the fluorophore azide is the limiting reactant, this means that many acyl sites of the starch ester will remain unmodified. For the starch ester, after 30 min the reaction solution appears clear as most of the fluorophore has been incorporated.

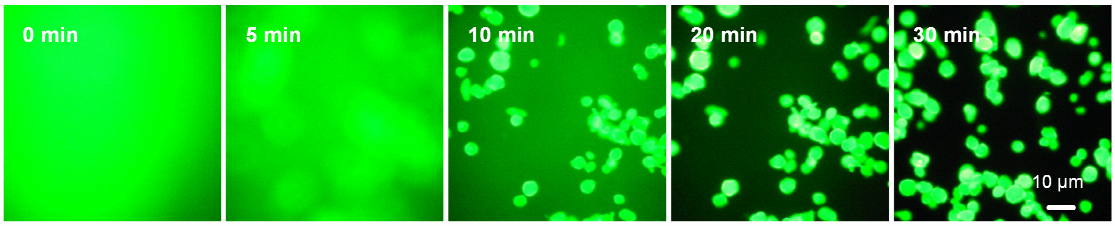

Figure 2. Fluorescence of click reaction solution with time for 10-undecynoic acid starch ester.

2.3 Click derivatisation of starch ester produced by catalysis with solubilised subtilisin

Subtilisin solubilised in isooctane was prepared according to literature[3]. Dry starch (20 mg in 1cm^3) was suspended in the isooctane/subtilisin solution or pure isooctane/AOT (Aerosol-OT or sodium bis(2-ethylhexyl) sulfosuccinate) for the non-enzymatic control) and methyl-5-hexynoate (threefold molar excess over hydroxyl groups in starch) is added. The mixture is incubated at 37 ^{o}C, mixed by inversion for 48 hours. The polysaccharide is washed in excess isooctane in order to remove unreacted methyl-5-hexynoate.

After click derivatisation of the starch product, fluorescence of the particles could be detected with fluorescence microscopy (Figure 3). In this case the non-enzymatic starch control showed no fluorescence, thus ruling out non-covalent binding of methyl-6-hexynoate during the enzymatic step, or azide during the click step.

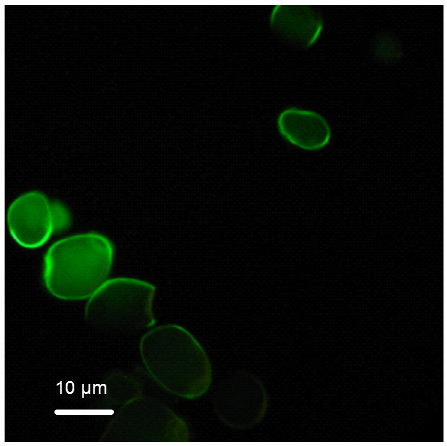

Figure 3. Fluorescence micrograph of starch-5-hexynoate, produced by solubilised subtilisin catalysis in isooctane.

2.4 Starch biotinylation and streptavidin attachment

The starch-streptavidin conjugate prepared with the above described conditions can be seen in the communication (Fig. 4).

The same experiment was performed with slightly altered conditions. The concentration of biotin azide in the click step was doubled, while the reaction time was increased to 180 min. Also the time that the streptavidin/Alexa Fluor 488 conjugate was allowed to attach to the biotinylated starch, in this case, was 30 min (as before in the dark). The other steps remained the same. The resulting product (Fig. 4) displayed fluorescence of higher homogeneity, though areas of higher or lower fluorescence were still visible. The increased homogeneity must be the result of increased streptavidin attachment to biotinylation sites that are not on the particles' outer surface, due to the increased reaction times and biotin initial concentration.

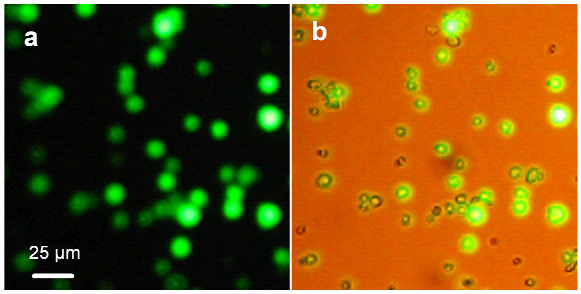

Figure 4. Fluorescence micrograph of starch/streptavidin derivative (alternative conditions), a: fluorescence image and b: merged fluorescence and transmission image

References

1 V. M. Paradkar and J. S. Dordick, *Biotechnology and Bioengineering*, 1994, 43, 529-540