Department of Pure and Applied Chemistry

ENZYMATIC ACYLATION OF STARCH

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Signed:
Date:

Research conducted for this dissertation was industrially funded by BASF and results should be treated as confidential until such time as confidentiality is waived and the University Library moratorium has expired.

To my parents

"...μὴ εἶναι βασιλικὴν ἀτραπὸν ἐπὶ γεωμετρίαν
-There is no royal road to Geometry"

Euclid

(from Proclus in his Commentary on the First Book of Euclid's Elements)

"And as you set out for Ithaca,

hope the road is long,

full of adventure, full of discovery…

…And if you find her poor, Ithaca has not deceived you.

Wise as you have become, with so much experience,

you must already have understood what these Ithacas mean."

Constantine P. Cavafy

Acknowledgments

I would like to acknowledge the contribution of certain individuals on the road to what has been my Ithaca for the past three hard years.

Firstly my home supervisor Prof. Peter Halling. His door has always been open to me and he was always available to provide guidance in matters of science or otherwise. It is rare one has the opportunity to meet, let alone work with, a true scientist and academic such as Peter. It has been a privilege. There has also been another source of guidance in the face of my second supervisor, Prof. Sabine Flitsch. Though in Manchester, Sabine was there (often through a webcam) with exciting ideas and views that have helped fuel this project.

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Marianna has been a constant source of support and has shown much patience, despite my inexplicable appetite for spending another three years doing "…something with enzymes!". I must also thank my brother Yiannis for sharing a home away from home with me and my sister Sophia.

Finally and most importantly I wish to thank my parents. Their guidance, support and love for the past 25 years have made all I have achieved, possible. They have helped me to become a better scientist, a better person.

Abstract

The possibility of enzyme catalysed synthesis of starch esters, is an extremely interesting prospect, as a new route for synthesis of biodegradable polymers from renewable material, through mild processes.

Starch esters have traditionally been analysed by saponification and back-titration, however this method was found to be unreliable. Quantification through integration of ^{1}H-NMR peaks, was also found problematic for low degrees of substitution (DS, mol acyl group per mol anhydroglucose). Therefore an analytical scheme employing alkaline methanolysis of starch esters and analysis of the resulting methyl esters by GC/FID is presented. This technique is employed for starch esters with fatty acids of medium chain length and can be suitably modified to accommodate other chain lengths.

Lipase catalysed synthesis of starch esters with decanoic acid was performed in concentrated aqueous systems, where the starch is gelatinised prior to enzymatic reaction. The reaction showed a pronounced optimal water content (1.25 mL of water for gelatinisation per g of starch), in which the equilibrium was sufficiently shifted, in order to produce starch acylated to a useful extent (0.018 DS).

Use of fatty acids with other groups on their alkyl chains, as substrates, led to successful synthesis of starch 10-undecynoate. The triple bond in the ω-position of the fatty acid may act as a grafting site for further modification, with azide containing molecules, through "click" reactions (dipolar cycloaddition).

Investigation of other reaction systems for enzymatic starch acylation, revealed non-enzymatic transesterification of vinyl decanoate with starch in DMSO. On the other hand, the ionic liquid 1-ethyl-3-methylimidazolium dicyanamide ([EMIM][DCA]), was a suitable medium for lipase catalysed synthesis of starch esters with decanoic acid (0.016 DS).

Treatment with α-amylase resulted in decreased hydrolysis for the modified starches, as the acyl groups present inhibited the action of the enzyme. The presence of approximately one acyl group for every one hundred glucose units is sufficient to decrease extent of hydrolysis by 11%.

Abbreviations

DS: Degree of substitution
Average number of substituted hydroxyls per anhydroglucose monomer (mol acyl per mol anhydrogluse, DSmax = 3)

AGU: Anhydroglucose unit

FAME: Fatty acid methyl ester

DAME: Decanoic acid methyl ester

DMSO: Dimethyl sulfoxide

DMF: Dimethylformamide

RRF: Relative Response Factor

CALB: *Candida antarctica* lipase B

RTIL (or IL): Room temperature ionic liquid
[EMIM]: 1-ethyl-3-methyl imidazolium
[BMIM]: 1-butyl-3-methyl imidazolium
[Ac]: Acetate
[DCA]: Dicyanamide

DNS: 3,5-Dinitrosalicylic acid

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Outline

The aim of the project, presented in this dissertation, is to investigate the possibility of employing enzyme catalysis to assist the acylation of starch. The possibility of producing a commercial material, from renewable and naturally abundant stock, through a mild process that can be termed "natural", is of extreme interest. The fact that this project was industrially funded (BASF), attests to this. But even from the purist's perspective, investigation of biocatalysis in systems of increased complexity, may assist in furthering the understanding of enzyme action in such circumstances. Biotransformation is one of those tools that has unknowingly been used for thousands of years and in modern times has found renewed interest, due to better understanding of the governing mechanisms. Biocatalysis sits on the interface of a number of fields and under its banner one will meet scientists and engineers of multiple disciplines.

In order to study the synthesis of any product, an acceptable bench-top analytical technique, which is fast and easy to perform is highly desirable. In the present case, this is necessary for the quantification of acylation of the reaction products. Due to the complex nature of starch the analysis of its esters can be a complicated process, despite the wealth of available literature on chemical acylation of starch. The most suitable technique may well depend on the particular application and starch product. After a general introduction, the first three chapters of this thesis, deal with the investigation into analysis. The shortcomings of established analytical techniques are discussed and a new analytical scheme, based on older approaches, is set up and adopted as a suitable method for use in the rest of the investigation.

The next four chapters deal with attempted enzymatic acylation with different approaches in each case. Different ways of bringing polysaccharide, enzyme and acyl donor into contact are investigated and the results presented. Ideally all three ingredients would be dissolved in a system with no competing nucleophile (e.g. water), where the enzyme remains active. This is highly unlikely, due to the insolubility of the hydrophobic acyl donor in polar solvents and the insolubility of the hydrophilic starch in almost everything. Furthermore even if a suitable system were available, contentedness of the enzyme is not guaranteed. In a suitable reaction system the substrate is usually either in a dissolved or suspended state. For starch

there is a third route. An interesting system where starch is in the form of a concentrated aqueous gel with fatty acid dispersed throughout is described. Next the possibility of biocatalysis with the polysaccharide in solution is investigated for the few solvents that are known to dissolve it. Dimethyl sulfoxide and ionic liquids are seperately discussed.

The final chapter deals with investigation into possible further characterisation of the products, prepared as described in the previous chapters.

Many other approaches for enzymatic catalysis of such reactions can be envisioned and some were attempted. However the experiments described throughout this thesis are the ones that yielded interesting results. Some experiments that one might expect to find in such an investigation, but yielded no conclusive result are described in the appendix .

After the general introduction with essential background information, the rest of the thesis is organised into chapters, styled in the fashion of research articles, with their own introduction, methodology and results sections. This may result in occasional repetition, but has the advantage that each chapter is relatively self-contained. This was found an extremely helpful and practical feature, in other theses with this layout, that were consulted by the author.

General Introduction

1. Starch: Nature's energy reserve

Starch is one of the most significant carbohydrates, mainly because of its nutritive importance in the human and animal diet. It is stored in granule form within the amyloplasts in several possible plant regions, from where it is taken when required to cover their energy needs. Starch is the most important final product of the photosynthetic process and unlike cellulose, the glucose units that form it can re-enter the metabolic track, after amylase assisted hydrolysis (or phosphorylase assisted phosphorolysis), in order to cover energy needs to synthesise other materials. Due to its attractive properties and low cost, through the years, starch has gained important roles in many industries. And even today while many traditional processes and materials are abandoned due to high costs or environmental concerns, starch shows promise for use in up and coming industries where properties such as biodegradability and renewability are very important.

1.1 Starch chemical structure

Starch granules differ in shape and size depending on their plant source, something that makes the identification of a specific starch type, an easy task. Starch consists of two types of glucose polymers, amylose and amylopectin, the ratio of the two depending on the source.

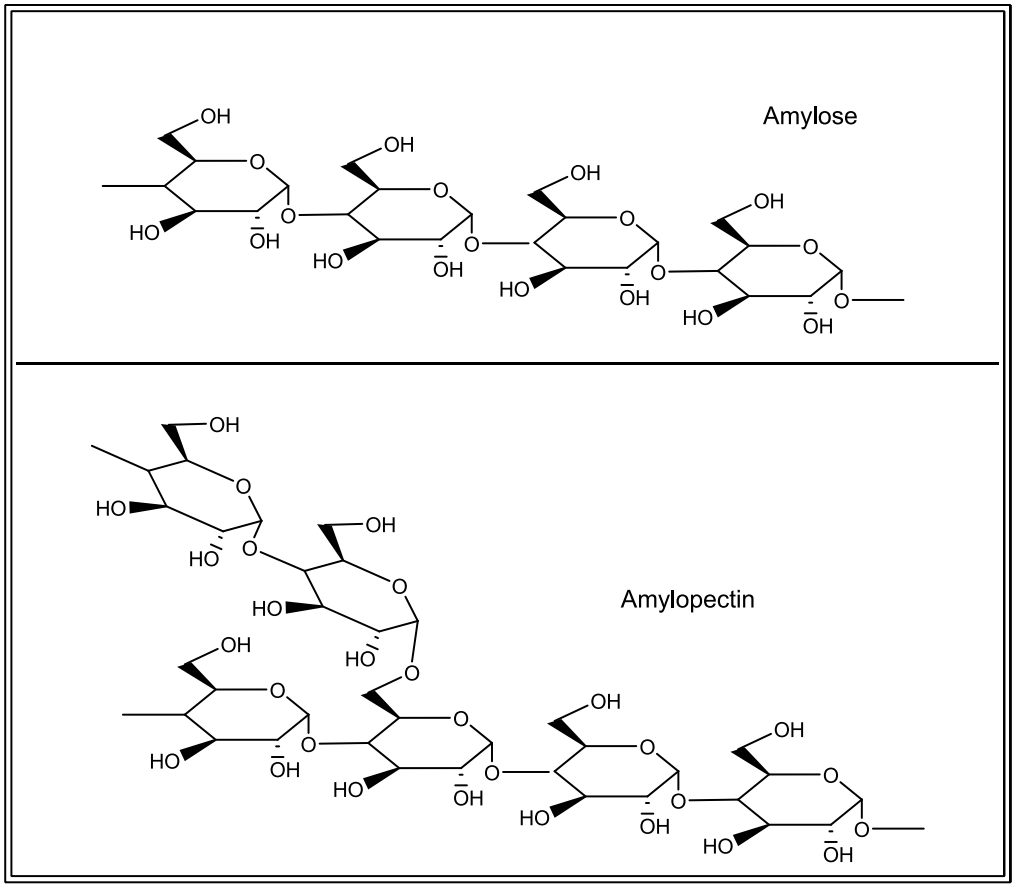

Figure 1. Linear amylose with α-1,4 bonded monomers and branched amylopectin with α-1,6 branches are the two types of glucose polymer found in starch granules

The linear chain amylose is situated in the granule interior and has a relatively low molecular weight, consisting of 100-2000 glucose molecules interconnected by α-D-(1→4) bonds. The anhydroglucose units throughout the amylose chain each contain one primary and two secondary hydroxyls. At one end of the chain the anhydroglucose unit also contains an aldehydic reducing group in the form of a hemiacetal (reducing end), while at the other end of the chain, the unit contains one primary and three secondary hydroxyls. The large amount of hydroxyls gives amylose an affinity for moisture and dispersibility in water. But, due to their linearity, amylose polymers have the tendency to align themselves in a parallel fashion and form hydrogen bonds between hydroxyls on neighbouring chains, thus enabling the formation of strong films. Amylose is the starch component responsible for the blue colour when starch is mixed with iodine. This is because of its affinity

for iodine as well as molecules containing a hydrophilic and hydrophobic segment, around which it forms a helical complex. The deep blue colour formed by the amylose-iodine complex can be used to identify amylose presence in starch and even quantitatively measure it, by colour intensity or potentiometrically. Amylose also forms complexes and precipitates with organic molecules containing hydrophilic moieties, thus enabling its separation from amylopectin by selective precipitation.

The branched chain amylopectin forms the exterior of the starch granule and has a molecular weight that ranges from hundreds of thousands to a couple of millions. The monomer glucose molecules connect with each other again through α-D-(1→4) bonds, in the main chains as well as the side chains (10-30 glucose residues), which connect to the main chains by α-D-(1→6) bonds (2-4%). This means that a single amylopectin molecule may have many non-reducing termini, but only one reducing end. Unlike amylose, amylopectin polymers can not easily align themselves to form hydrogen bonds, because of their size and branched structure. This results in clear and stable amylopectin aqueous sols that do not favor the formation of strong films.

In some starch types, namely grain starches, investigators have detected another type of a-glucan. This is of intermediate molecular weight and is termed as anomalous amylose, or anomalous amylopectin. Other researchers, however, (Tester & Karkalas, 1996; Tester et al., 2004) dispute the existence of that intermediate material, but accept that in some starch mutations exists a type of soluble starch, similar to animal glycogen. For this reason it was named phytoglycogen (Ball *et al.*, 1996; Wong *et al.*, 2003).

The use of debranching enzymes followed by size exclusion chromatography is one of the main methods used to gain conclusions about starch chemical structure. This technique led to the conclusion that amylose chains actually do present a very small number of branches, which differs according to the starch source (Takeda *et al.*, 1986; Takeda *et al.*, 1984).

Similar studies on amylopectin structure contributed to the formation of the “cluster” model (Manners, 1989). According to this model each molecule consists of three classes of glucose chains named A, B and C. The A chains bind in clusters to B chains, which in turn bind to other B chains or the C chain, of which there is only one per molecule. The C chain is the one which contains the reducing end of the

amylopectin molecule. Because of this complicated structure there is room for a lot more variation in respect to the chain lengths and branching patterns, compared to amylose.

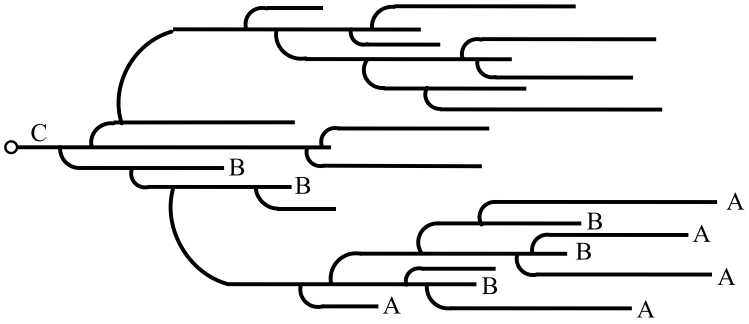

Figure 2. Cluster type architecture of amylopectin in the starch granule

Usually the ratio of amylose in starch varies from 20-30%, for common types such as wheat, potato, tapioca and corn. There are starches, derived from genetic modification that consist of much higher or lower amounts of amylose. "Waxy" starches contain hardly any amylose at all, while high amylose (or amylo-) starches, as there name denotes, contain up to 70% amylose.

1.2 Granule structure

As mentioned before, starch molecules are organised into a granule structure. It is accepted that these granules consist of amorphous and crystalline areas (French, 1984). The short-chained amylopectin molecules can be arranged into double helices (as proven by ^{13}C NMR), most of which in turn form crystalline lamellae (or crystallites). These crystallites, combined with remaining helices, form the ordered part of the molecule, while the amorphous part is believed to consist of amylose and long chained amylopectin. The semi-crystalline and amorphous parts are then ordered in alternate layers. Use of light microscopy with cross-polarisers, proves the presence of crystallites in the starch granules as they exhibit birefringence. The interference pattern obtained is that of a maltese cross, which indicates an orderly arrangement of the crystalline area within the granule (Wang *et al.*, 1998).

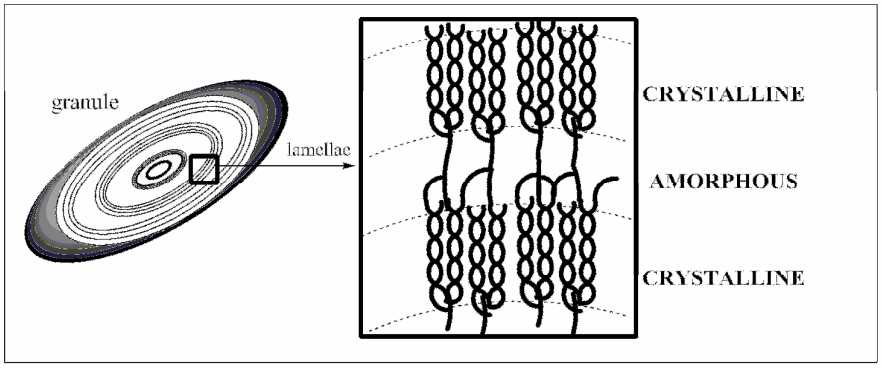

Figure 3. Arrangement of amylopectin and amylose in the potato starch granule.

Oostergetel and van Bruggen (Oostergetel & Vanbruggen, 1993), proposed a three-dimensional model for the organisation of crystallites in potato starch. According to this the short chain amylopectin double helices are approximately 5 nm long and are arranged into 5 nm thick lamellae. The lamellae alternate with the amorphous regions where the α-(1,6) branch points occur, forming on the whole a mostly continuous super helical structure. The helices that form the lamellae are organised in polymorph structure, termed polymorph B, in the case of potato starch. Other starches exhibit the A polymorph (e.g. maize), the main difference being that the A structure is more dense and binds less water within the crystalline area. Some starches such as certain legume starches, exhibit both types of polymorphs and thus are termed C type starches (Wang *et al.*, 1998).

1.3 Starch Gelatinisation

When heated above a characteristic temperature in an excess of water the starch granule swells to many times its original size and loses its crystalline order. This temperature is called the gelatinisation temperature and the phenomenon, gelatinisation. X-ray scattering techniques revealed that during gelatinisation the amorphous areas of the starch granule allow water ingression thus swelling. As this happens, a stress is imposed on the crystalline areas through the amylopectinic material connecting the two regions. The build-up of stress results in the dissociation of the amylopectin double helices and the rupturing of the granule structure (Donald, 2001).

Though, once past the gelatinisation point, this is not a reversible process, gelatinised starches do tend to retrograde when cooled. The polymer molecules will not reform a granule but they will realign themselves into a more arranged structure, with hydrogen bond formation between linear parts of the molecules (this is especially the case for linear amylose). This causes the starch solution to become more viscous and gel. Syneresis, that is expulsion of water from the polymer network, can be observed in some cases, during this process.

Starch gelatinisation is commonly used as pre-treatment when the glucan chains need to be made more readily available to other reactants in chemical modifications.

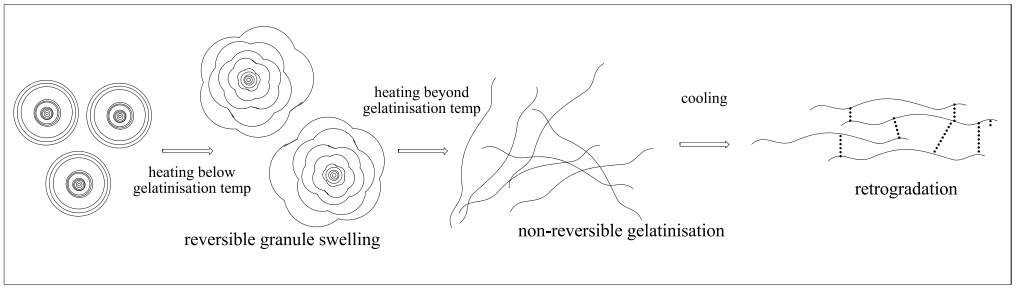

Figure 4. Starch gelatinisation: Granules suspended in water are initially swollen to many times their size before they reach gelatinisation temperature and rupture occurs. The free chains realign with cooling of the mixture during retrogradation.

2. Modified starches

Although native unmodified starches show promise for a number of applications, certain physical properties they and their aqueous sols exhibit, limit greatly their usefulness. The fact that starch is insoluble and does not swell in cold water, the difficulty to control viscosity after and during cooking, sensitivity and tendency to break-down during excessive cooking, lack of clarity of starch sols and the tendency to become opaque and gel when cooled (Wurzburg, 1986c), the lack of mechanical integrity of native starch films due to small flexibility, tensile strength and elongation of amylopectin (Cheyne *et al.*, 2001), processing difficulties during extrusion or injection moulding in the plastic industry (Aburto *et al.*, 1999) and other negative starch properties, drove researchers to attempt modification of starch in order to produce substances with improved properties. This allowed the expansion of starch's usefulness for a myriad of industrial applications. The desired properties for the modified starch depend on the actual application. For example, in the food industry starch derivatives may be required to have any one of a number of different properties such as gelled, flowable, expanded, rigid, rubbery etc. (Langan, 1986).

Any induced alteration of the chemical and/or physical properties of starch, can be considered as starch modification (Wurzburg, 1986c). Bearing this in mind one can distinguish three main separate routes for starch modification. The first is the modification of physical properties of starch through non-chemical processes, which results in products such as pregelatinised starches, redried starches, extruded starches etc. The larger trend is the one involving chemical modification of starch. Popular products include chlorinated, pyroconverted, cross-linked, acylated starches and many other derivatives where substitutent groups are introduced into the starch molecule. Finally a more recent completely separate trend is the genetic engineering of plants, aimed at altering metabolic pathways, in order to obtain native modified starches.

The traditional industries with high demands for modified starches are the food industry, the paper industry, the textile industry and the corrugating and adhesives industries (Wurzburg, 1986c), but new types of purer modified starches seem very

promising for use in emerging or even older industries such as the biomedical, the pharmaceutical or the biodegradable materials industries.

2.1 Physical Modification

Physical modification for many years lay in the shadow of popular chemical methods for starch modification. In recent years however there has been increasing interest in physical modification using heat, moisture, shear, radiation or a combination of these, as the products are considered natural materials and find uses in food applications (Jacobs & Delcour, 1998). Many techniques for physical modification of starch are used in combination with chemical methods, or even as pre-treatment to make chemical modification easier.

One of the main setbacks for the use of starches as thickeners in the food industry, is the fact that they do not adopt desired properties before they have first been cooked and left to cool. A popular convenience product addressing this issue is pre-gelatinised starches, used in many packaged premixes as a viscosity agent. These modified starches are basically precooked to above their gelatinisation temperature and dried, so that when added to cold water they form viscous pastes. The drying techniques differ but the main ones used are spray-drying, roll-drying and extrusion or drum-drying. The different particle sizes produced by different drying methods, are responsible for slight differences in pasting properties between products. Smaller particles give thicker (but lumpier if not properly prepared) pastes, while larger particles result in low-viscosity and grainier pastes (Maywald Snyder, 1984).

Other types of hydrothermally modified starches are annealed starches and heat-moisture treated starches, where the granule structure is not affected but the behavior during cooking is altered. Annealing involves treating starch in excess water (above 40%w/w), above the glass transition temperature (varies, but typically 20-40^{o}C) but below the gelatinisation temperature (typically 60-80^{o}C). Treating starch at the same temperatures but at lower moisture levels (below 35%w/w), is the principle of heat-moisture treatment. The first method results in higher but shorter gelatinisation temperature ranges, while the latter produces starches with lower but broader gelatinisation temperature ranges (Jacobs & Delcour, 1998). These techniques can be

used to obtain starches with desired properties, such as more stable viscosities during cooking.

Starch may obtain a more flowable and expanding structure through extrusion with water at high temperatures. During this process, the weakest particles with the highest water content are destroyed and lubricate the flow of drier, harder remaining particles (Cheyne *et al.*, 2001). But use of native starches is difficult at high temperatures, because of their tendency to break down, so chemically cross-linked starches are preferred (Langan, 1986).

Radiation is rarely used by itself and is usually combined with a chemical technique for starch modification.

2.2 Chemical Modification

For many industrial applications only a low level of chemical modification is actually needed to suitably alter the properties of the native starch. This makes chemical modification a very attractive approach to starch derivative production. Most commercial starches have a degree of substitution (DS) less than 0.2, where **degree of substitution is the average number of sites per anhydro glucose unit on which there are substituent groups**. The maximum degree of substitution is 3, as there are 3 hydroxyls that can be substituted on each monomer in the glucan chain. High levels of substitution totally alter the properties of starch (Sagar & Merrill, 1995).

2.2.1 Converted starch

The first attempts to improve the properties of starch through chemical modification, concentrated on producing a derivative that could be cooked at higher concentrations, while remaining clear and flowable. This was succeeded by causing the rupturing of starch granules, thus minimising swelling and thickening during heating. This kept starch viscosity low, allowing larger quantities of starch (more concentrated dispersions) to be used at once. There have been a number of different techniques used to produce converted starch (as this derivative is called).

During acid conversion, a starch slurry is heated below its gelatinisation point and treated with mineral acid (e.g. hydrochloric acid, sulphuric acid etc.) while agitated

for a period of one to several hours. The starch granules are then recovered after the acid is neutralised (Chamberlain & Rao, 1999). During this process the acid hydrolyses glucosidic linkages in the a-glucan chains, attacking the more accessible amorphous regions at first and then the crystalline ones.

Converting starch through treatment with oxidising agents is mainly aimed at researching the chemical structure of starch. In industry it is used only for bleaching and sterilising starch in order to remove impurities. Oxidising techniques that lead to changes in the chemical nature of starch involve the use of halogens (usually chlorine or bromine) or hypochlorite. These converted starches are often referred to as chlorinated starches, which is a misnomer as they are merely oxidised and not actually chlorinated.

Pyroconversion is another important technique used to produce converted starches, and consists of four major steps, pre-treatment (acidification or buffering), predrying, heating or pyroconverting and cooling. The products of this method fall into three categories, namely british gums, white dextrins and yellow or canary dextrins. The differences between the final products are the result of differences in the pre-treatment and the extent of heating during the pyroconversion (Wurzburg, 1986a).

2.2.2 Cross-linked starch

In many products it is required that starch keeps its viscosity past the gelatinisation point. This is not the case with native starch as the hydrogen bonds holding the granules together fall apart and the structure collapses, giving the sol a cohesive rubbery texture, sensitive to acids or mechanical shear. In order to keep the granule structure together and maintain a stable viscosity, chemical cross-linking of starch is employed, through the use of bi- or multi-functional chemicals, which are chemicals containing two or more moieties capable of reacting with hydroxyl groups. These reagents form chemical bridges between monomers on the same or different molecules, strengthening the granule structure and preserving it during heating, even when the hydrogen bonds are destroyed. Though many reagents are capable of forming these bonds, only a few have gained commercial significance, namely adipic acetic mixed anhydride which forms distarch adipates, phosphorus oxychloride which forms distarch phosphates and epichlorohydrin forming distarch glycerols.

There is also been plenty of research into the possibility of cross-linking starch to other polymers with hydroxyl groups, such us cellulose (Wurzburg, 1986b).

2.2.3 Starch ethers

The discovery that starch can react with alkylene oxides in aqueous slurries, under carefully regulated conditions, while keeping the granule intact thus enabling it to be retrieved by simple washing techniques, propelled research into the commercial value of starch alkyl-ethers.

In industries such as detergents, adhesives and textiles the tendency of starch aqueous pastes to thicken and gel when cooled is highly undesirable. The need is for starch to maintain a stable viscosity, as rapid changes in viscosity cause irregularities in the final products. Oxidising starch will result in a derivative with stable but decreased viscosity while the carboxyl groups introduced by the reaction make it unsuitable for most applications, as they reduce its affinity for negatively charged substrates. For this reason hydroxyethylated starch has been introduced as the answer to these demands. This low-cost derivative has high viscosity stability while the ether bond remains stable to hydrolysis even at elevated pH conditions (Jauregui *et al.*, 1995). Hydroxyethylated starches though have not been approved for use as food additives. The gap for a modified starches with stable viscosity, needed as additives in food application where they would undergo freeze/thaw cycles and be stored over long periods, was filled by the product of starch reaction with propylene oxide. Hydroxypropylated starches have been approved for use as food additives, but have a much elevated cost thus limiting their commercial applications to that (Moser, 1986). Other starch alkyl ethers such as cyanoethyl starch, have been introduced, but have failed to obtain approvals for use in sensitive industries, thus leaving hydroxyethylated and hydroxypropylated starches to be by far the most commercially important starch alkyl ethers.

The paper industry uses starch as wet end additive, surface sizer and coating binder, while cellulose is the main substrate. Cellulosic fibres are negatively charged, so a starch derivative with an affinity for such substrates gains significant commercial importance in paper making. Such starches, called cationic, are produced by chemical reaction with amino, imino, ammonium, sulfonium or phosphonium

containing reagents, thus substituting the starch chain with groups that can carry positive charges. These derivatives improve sheet strength mainly through ionic bonding between the modified starches and the cellulose fibres (Solarek, 1986). The most commercially important such starch derivatives are tertiary amino and quartenary amino starch ethers, but substrates such as 2-diethylaminoethyl chloride have been introduced recently in order to obtain products that can be used in papers that will come in contact with food.

2.2.4 Starch co-polymers

Many industries using petrochemicals have been forced recently to turn their attention to other substrates, due to large consumption and exhaustion of natural resources. The polymer/plastics industries saw starch as a promising cheap and abundant natural resource suitable to replace traditional polymers. Though starch lacks the mechanical properties on its own, when grafted with other polymers to form copolymers, it obtains important properties (Fanta & Doane, 1986). Another bonus starch presents is its biodegradability, thus combating important issues on plastics eco-friendliness and waste management. Thermoplastic starch and starch copolymers with poly(vinyl acetate), poly(ε-caprolactone), poly(D,L-lactic acid), polyhydroxyalkanoates and poly(butylenes succinate-co-butylene adipate) are the most commercially important products derived from this approach (Wang et al., 2003).

2.2.5 Acylated Starches

Many types of starch esters have been prepared and have found uses in a wide range of applications. The hydroxyl groups of the anhydro glucose monomers, offer a site suitable for esterification with acyl group containing substrates. Acetylated starches have been around for many years and low DS derivatives are used in the food industry in order to control and adjust the rheological behaviour of pastes (Jarowenko, 1986). Succinate starches are another popular group of esterified starches with great commercial importance. Starch succinates reinforce swelling capability at lower temperatures (Rudnik *et al.*, 2005), while alkenyl succinate starch derivatives, introduce hydrophobic groups to the hydrophilic chain giving the starch

emulsifying capabilities. Fatty acid starch esters are used in starch copolymerisation, as it has been noted that fatty acid ester acts as an internal plasticizer (Thiebaud *et al.*, 1997), while recently their potential use in drug delivery systems and other biomedical applications is being investigated (Malafaya *et al.*, 2001). In order to obtain the starch esters with carboxylic acids, the most popular approach involves use of the acid anhydride or acid chloride (more effective for higher esters), in the presence of pyridine which acts both as catalyst and solvent (Sagar & Merrill, 1995). These reactions can proceed with the starch in suspension or gelatinised, but generally show low selectivity. This can sometimes be controlled with use of different reagents or conditions (Tomasik & Schilling, 2004).

3. Enzymes: Nature's own catalysts

Enzymes are the catalysts that living organisms use to accelerate reactions that are required to take place within their cells. They are responsible for the catalysis of metabolic pathways as well as many cell regulatory mechanisms. The vast majority of enzymes are protein type molecules, except for some catalytically active ribonucleic acids (ribozymes). Being nature's catalysts, with their design constantly improved through evolution over the course of billions of years, enzymes usually are very specific towards particular substrates and particular reactions and they are extremely effective catalysts as they can increase reaction rates by a factor of 10^{12} or even more. The chain of aminoacids that compose enzymes is folded in a three dimensional structure, predominantly determined by the monomer sequence. Usually hydrophilic moieties are located on the outer surface of the enzyme towards the aqueous environment, while hydrophobic groups tend to be protected within the structure (this is not absolute, in many enzymes a lot of hydrophobic surface can be present). This results in the hydration of the enzyme and interaction of water molecules with the different parts of the protein's structure. There is a detectable change in the properties of the water molecules, depending on the level of interaction with the enzyme (Kurkal-Siebert *et al.*, 2006; Ohno *et al.*, 2001; Pocker, 2000). The whole structure is supported by weak binding forces (disulfide bridges are the only covalent bonds) between spatially adjacent monomeric units. This results in a delicate structure that can be unstable in solution, leading to loss of active conformation due to unfavourable conditions, e.g. temperature, pH, salt concentrations.

There have been many models attempting to describe the action of enzymes on substrates, but Fischer's "Lock-and-Key" mechanism and the Induced-Fit mechanism, illustrated as the interaction of a hand (substrate) and glove (enzyme), are amongst the most popular illustrative models available (Faber, 2004b). Though specific enzyme mechanism can be extremely complicated and may vary from enzyme to enzyme, the stabilisation of the reactants in a transition state is the shared key step in all enzymatic catalysis (Koolman & Roehm, 2005).

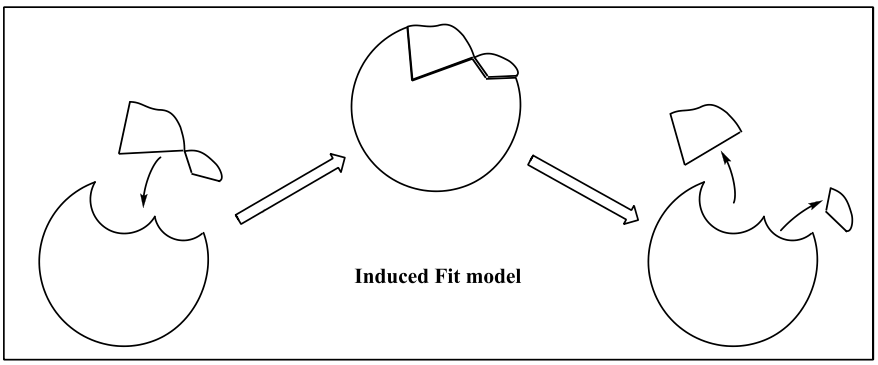

Figure 5. The induced fit model suggested that the active site of the enzyme is not an exact complementary shape to the substrate (lock-key model), but rather can change shape on approach of substrate to accommodate it. This improved model explains the need for certain structural features of the substrate apart from the reactive group.

3.1 Enzymes in organic chemistry

Despite their obvious advantages enzymes have traditionally been prejudiced against in organic synthetic chemistry, due to certain deep-held beliefs about their action (Faber, 2004b; Klibanov, 1990). Often organic chemists consider enzymes to be too expensive, sensitive, specific and only active in their natural environment. These statements, though based on truth, are simplistic, outdated and may be misleading as developments in enzyme science for the past twenty or so years has shown. Enzymes are now known to be remarkably stable if certain conditions are met, cheap in many cases especially when produced on larger scales, recyclable further reducing costs, in many cases quite promiscuous towards the substrate and are known to remain catalytically active in non-aqueous environments. Ergo, it is obvious that under certain circumstances, enzymatic catalysis may be the better or even the only route for an organic reaction. In the table below the advantages and disadvantages of enzyme use for catalysis of organic reactions are summarised (Bornscheuer & Buchholz, 2005; Carrea & Riva, 2000; Faber, 2004b; Loughlin, 2000).

Advantages	Disadvantages
• Enzymes display higher acceleration of reaction rate compared to traditional catalysts. This means that less catalyst is usually employed in enzymatic systems • Enzymes are extremely chemo-, regio- and enantio-selective, resulting in minimal by-product formation • Mild reaction conditions required (close to room temperature and physiological pH) • Enzymatic reactions are considered "natural processes" • Enzymes are usually compatible with each other and can be used in one-pot reaction schemes • Enzymes are tolerant towards unnatural substrate, reaction and environment • Diversity of available enzymes, means they can catalyse most organic reactions and some that are not possible non-enzymatically (e.g. selective hydroxylation of aliphatics)	• Only one enantiomeric form of an enzyme is provided by nature • Sometimes laborious and difficult isolation of enzymes • Natural cofactors can not be replaced • They are most active in water • High substrate or product concentrations may lead to inhibition

Table 1. Advantages and disadvantages of enzymes as catalysts for organic chemistry

3.2 Hydrolytic enzymes

Enzymes are divided into general groups, called enzyme classes, depending on the type of reaction they catalyse. The class of enzymes that has received most attention from the scientific community, for biocatalysis, in recent years is the hydrolases, which catalyse the hydrolysis of chemical bonds. The lack of sensitive cofactors, the

number of available enzymes and their relatively promiscuous nature towards the substrate are the main reasons for this (Bordusa, 2002; Jaeger & Eggert, 2002). Proteases, esterases and lipases - the catalysts for amide and ester bond hydrolysis - are the most popular hydrolases, amounting for about ⅔ of the biotransformations reported in research (Faber, 2004a). It is common for the mechanism of action for these enzymes, to begin with attack of the carbonyl group in the substrate by a nucleophilic moiety in the enzyme active site. In serine hydrolases (Kazlauskas & Weber, 1998), the hydroxyl group of this particular aminoacid acts as the nucleophile. The serine is accompanied by two adjacent aminoacids (aspartic acid and histidine) forming the "catalytic triad". This arrangement activates the serine, promoting a nucleophilic attack on the substrate's carbonyl group, resulting in the "acyl-enzyme" intermediate. This can in turn be attacked by another nucleophile (water in hydrolysis), resulting in release of the product and regeneration of the catalyst.

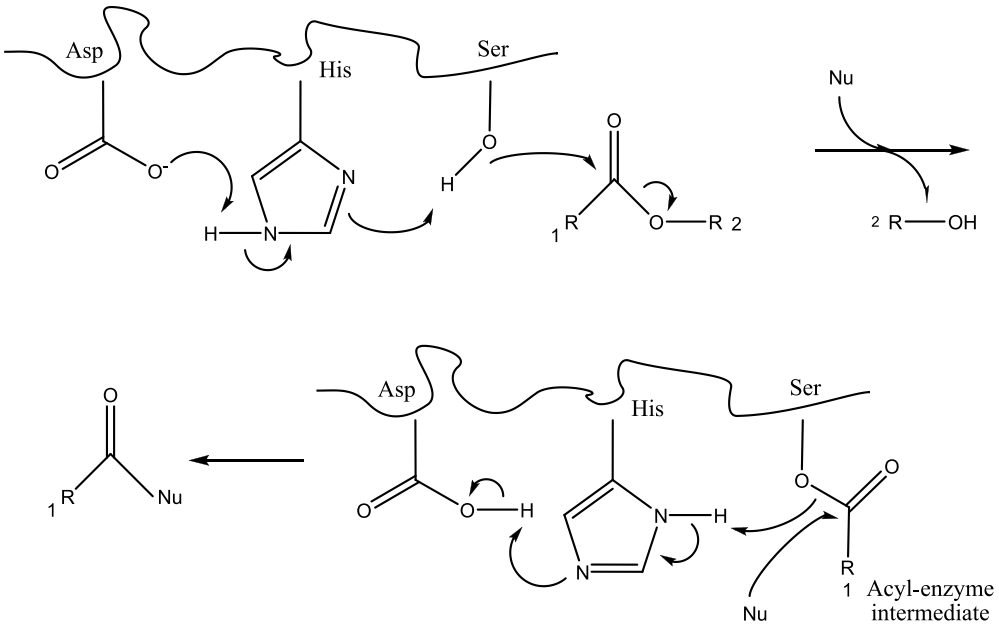

Figure 6. Mechanism of action for serine type hydrolases on an ester

3.3 Enzymes in low water systems

As previously mentioned, water plays an important and complicated role in biocatalysis, being the natural environment of most enzymes. It partakes in non-covalent interactions necessary for catalytic activity, while at the same time is needed

for most protein denaturation reactions (Zaks & Klibanov, 1984). Therefore hydration is required for the enzyme to remain catalytically active, while total dehydration is considered to render enzymes inactive. However it seems very low levels of hydration are sufficient to activate enzymes (Loughlin, 2000; Pocker, 2000) Therefore, it can be perceived that if the water required for retention of activity is present, the enzyme may remain active in other solvents.

Indeed it is known that a number of enzymes are naturally found in lipophilic environments (membranes), but even enzymes that work in water have been reported to retain their catalytic activity in organic solvents. Interestingly enough the use of enzymes in organic solvents may even impart improved properties to the enzymes (Klibanov, 1997; Zaks & Klibanov, 1985, 1986). Enzymes are known to be more stable, since the water that is required for the cascade of inactivation steps is not available, e.g. stability of porcine pancreatic lipase at 100^{o}C for several hours in 99% organic solvent was the first report of such behaviour (Zaks & Klibanov, 1984). It has also been observed that specificity and selectivity of biocatalysts can be manipulated by variation of organic solvent, this is probably due to increased rigidity of the enzymatic structure as a result of lack of water's action as a "molecular lubricant" (Broos *et al.*, 1995) aiding in protein conformational changes (Zaks & Klibanov, 1986).

However probably the most important useful effect of organic solvents on enzymatic action, is the reversal of thermodynamic equilibrium for hydrolases, from hydrolysis toward synthesis. After the formation of the "acyl-enzyme intermediate" in hydrolase catalysed reactions, if water content is low then another nucleophile can compete in accepting the acyl group (Adachi & Kobayashi, 2005). This leads to formation of esters, amides etc when hydrolases are used in systems where little water is available, if their catalytic activity is retained.

Use of enzymes in non-aqueous media has been of great interest and a number of aspects of their behaviour in such systems is now understood, at least to an extent that reliable predictions can be made (Bell et al., 1995; Carrea & Riva, 2000; Gupta & Roy, 2004; Klibanov, 1990). Organic solvents have long been established as the standard medium for organic chemistry over water, therefore the possibility of

performing biotransformations in such systems generates new potential for use of enzymes as tools in organic synthesis.

3.4 Enzymatic acylation of mono- and oligo-saccharides

Carbohydrates are a type of naturally occuring aldehyde or ketone, with a number of hydroxyl groups attached. Their esters with fatty acids have been found to possess amphiphilic properties, making them popular compounds for the pharmaceutical, cosmetic and food industries where they can be used as nonionic surfactants (Degn *et al.*, 2000; Ganske & Bornscheuer, 2005a; Otto *et al.*, 1998a, b).

However due to the presence of many hydroxyl groups, selective synthesis of carbohydrate esters with carboxylic acids can be a complicated process, requiring many protection-deprotection steps (Lindhorst, 2007b; Therisod & Klibanov, 1986). The potential use of enzymatic catalysis in these types of reactions could circumvent a large number of steps and many synthetic difficulties. Indeed the synthesis of monosaccharide esters, catalysed by hydrolases was one of the early examples of enzymatic synthesis in organic solvents. Therisod and Klibanov (Therisod & Klibanov, 1986) documented the successful transesterification of trichloroethyl esters of aliphatic carboxylic acids with glucose, galactose, mannose and fructose, carried out in pyridine with catalysis by porcine pancreatic lipase. Furthermore the authors reported that the acylation showed high selectivity towards the primary hydroxyls, even reaching 100%. The same group followed this up, by reporting the possibility of selectively producing regiomers of glucose fatty acid esters on secondary hydroxyls, after protection of the primary hydroxyl. Different enzymes showed selectivity towards different secondary hydroxyls (Therisod & Klibanov, 1987). In addition to catalysis of regioselective acylations, enzymes are employed for selective deprotection of fully acylated sugars (Lindhorst, 2007a).

However, there are drawbacks for these reactions. There are few organic solvents that will dissolve both the polar sugars and non-polar fatty acids, e.g. DMF, DMSO, pyridine. Furthermore these solvents present unfavourable environments to most hydrolases and are usually considered hazardous in the more sensitive industries (food, pharmaceuticals) (Riva *et al.*, 1988). The lack in sugar solubility in particular, becomes an increasing problem when the reaction is employed to oligosaccharides of

increasing size (Perez-Victoria & Morales, 2006; Riva *et al.*, 1988; Riva *et al.*, 1998). This suggests that other systems altogether may be required for large carbohydrates.

Other approaches involved using monosaccharide derivatives with increased solubility in less polar organic solvents (Bjorkling *et al.*, 1991), employing solid-phase systems (Cao *et al.*, 1996) and using novel types of solvents such as ionic liquids (Ganske & Bornscheuer, 2005a, b) or supercritical carbon dioxide (Heo *et al.*, 2000). Each of these overcame previous issues to a certain extent, but are not without their own setbacks. In any case though, the use of enzymatic catalysis to selectively synthesise products from natural sources such as carbohydrates remains an extremely interesting and useful process.

4. Enzymatic modification of starch

Enzyme catalysed reactions to modify starch can be considered as a subcategory of chemical modification, but even though the theoretic range of possible applications is massive, the only heavily researched area up to now is enzymatic hydrolysis.

Enzyme catalysed hydrolysis of starch is considered a very important alternative to acid treatment, as a route to obtain starch oligomers that find commercial application, such as dextrins, maltose or glucose. Enzymes offer the advantage of purer final product, with very few by-products, due to their high specificity and selectivity. There is a large number of different starch degrading enzymes whose use in such applications has been studied in order to obtain different final products with different functionalities. Popular enzymes are different types of α-amylases, β-amylases, glucoamylases (or γ-amylases or amyloglucosidases) and pullulanases (see Chapter 8).

As discussed, enzyme use in organic synthesis has been the focus of much attention, after the discovery that hydrolytic enzymes can be used in organic media to catalyse synthetic reactions, and the use of carbohydrates for this type of reactions has been successful. Mono- and oligo-saccharides are low-cost substrates whose derivatives may have a high commercial value and a very broad range of applications (Bordusa, 2002; Schmid & Verger, 1998). Because of low sugar solubility in organic solvents, however, there is always a compromise to be made between the enzyme synthetic activity and sugar solubility. For this reason there are few examples of products made for tri- and tetra-saccharides (Riva *et al.*, 1988, 1998; Perez-Victoria and Morales, 2006). It follows that the use of polysaccharides is even more challenging due to their very low solubility, especially in hydrophobic solvents.

However, the commercial importance of modified starches makes researchers constantly seek alternative methods of production that are more efficient. Furthermore, novel possible uses of starch derivatives in sensitive industries, such as the pharmaceutical and biomedical industries, require very mild production processes. Enzymes require mild conditions and their selectivity means that products of higher specificity can be produced, in comparison to chemical methods that usually require harsh conditions and chemicals.

4.1 Published work on enzymatic acylation of starch

Only recently has there been interest into possible use of enzymes as catalysts for reactions that substitute the hydroxyl groups of the starch anhydroglucose monomers, while there is only one earlier publication addressing this issue that was not followed up. The first publication was in 1995 (Bruno *et al.*, 1995), where the researchers used amylose, rather than starch, as a substrate. In the last few years three groups have published work examining enzyme catalysed acylation of starch (Chakraborty *et al.*, 2005; Qiao *et al.*, 2006; Rajan & Abraham, 2006; Rajan *et al.*, 2006; Rajan *et al.*, 2008). All groups adopt different approaches to the matter depending on their own background. Chakraborty *et al.*, showed interest in attaching groups that would act as a graft where another polymer could then bond or form. Rajan and Abraham focused on the use of substrates native to their venue of research, finding possible uses for local products and by-products. Qiao *et al.*, looked to extend the possible uses of commercial alkyl and alkenyl ketene dimers.

4.1.1 Solubilised enzyme

Dordick and coworkers (Bruno *et al.*, 1995) showed interest in the process of solubilising enzymes in organic solvents, by coating them with a surfactant. This approach had worked for monosaccharides and they attempted to use it for polysaccharides. The starch form used was a thin layer of amylose deposited on ZnSe slides, the reaction medium was iso-octane and the solubilised enzyme was subtilisin Carlsberg. They reported a DS of 0.15 for vinyl caprate with substitution occurring selectively for the hydroxyl of the 6 position of the anhydro glucose moieties. They also reported no enzymatic reaction for enzymes in non solubilised form.

4.1.2 AOT coated starch nanocrystals

The incorporation of enzymes into reverse micelles with use of surfactants is a well researched concept. Aerosol-OT (AOT, bis(2-ethylhexyl)sodium sulfosuccinate), is a popular surfactant that forms a monolayer around water droplets, thermodynamically stabilising them in oil. Proteases in reverse micelles were active when used for the modification of insoluble amylose in organic solvents, but the extent of the

modification was greatly limited by the inabillity of the enzymes to diffuse into the substrates (Bruno *et al.*, 1995). Gross and co-workers (Chakraborty *et al.*, 2005) investigated the possibility of encapsulating commercial starch with an average size of 40 nm, into AOT coated droplets. They successfully created AOT coated starch nanoparticles, by adding aqueous starch nanoparticle solution dropwise to AOT/anhydrous isooctane and then drying the micelles that formed. They also succeeded to a lesser degree with other surfactants such as Triton X-100 and CTAB (hexadecyltrimethylammonium bromide or centrimonium bromide). These nanoparticles were then used as substrate for lipase catalysed acylation reactions. The substrates chosen as acyl donors, were fatty acid vinyl esters that increase starch hydrophobicity, maleic anhydride which forms a polymer grafting or cross-linking site and epsilon-caprolactone which also can ring-open to form polyester grafts. The lipase used was *Candida antarctica* Lipase B, in immobilised (Novozym 435) and free (SP-525) form. The reactions all took place in round-bottomed flasks where the coated nanoparticles were added to organic solvent (in which the nanoparticles apperared to dissolve) and the immobilised enzyme, while stirred and incubated. In the case of the free enzyme it was co-encapsulated with the starch nanoparticles. The effects of incubation temperature and time were also studied. ^{1}H and ^{13}C-DEPT-135 NMR methods were used to analyse the results and calculate reaction efficiency (bound substrate/total subtrate) and DS. Vinyl stearate was reported to be the most efficient fatty acid vinyl ester substrate (DS=0.8), while free stearic acid did not react. A trend favouring longer chained acid vinyl esters was clear from the results obtained. ε-Caprolactone resulted in a maximum DS of 0.6, but substrate added after this point did polymerise onto the already substituted groups. For maleic anhydride a DS of 0.4, is reported. Authors stated that the hydroxyls substituted were only the ones linked to the C-6 of the glucose units, in opposition to similar products derived from chemical methods were the regio-selectivity could not be controlled (Choi *et al.*, 1999).

4.1.3 Native starch-free fatty acid esterification

Rajan and Abraham (Rajan & Abraham, 2006; Rajan *et al.*, 2006; Rajan *et al.*, 2008), focused their line of research on the possibility of assisting lipase catalysed fatty acid

acylations of native cassava starch and maize starch, by microwave radiation. They also investigated possible reactions in liquid and semi-solid state. The enzymes used were commercially important enzymes, much researched for organic synthesis reactions (lipolase and Amano lipase PS). The acyl donor was hydrolysed coconut oil, containing a mixture of different free fatty acids. The liquid state reactions were done in DMSO in which starch is highly dispersed, but which also is reported to strip essential water molecules from enzymes thus deactivating them. The lipolase reaction was carried out with a large ratio of water present, while the Amano PS lipase reaction was carried out in non-aqueous conditions. In the semi-solid state reaction carried out, gelatinised starch was used and the surfactant Triton-X 100 was present. Microwave reactions were carried out at low power (80 W) with small intermittent heating cycles. Microwave radiation has previously been used to assist non-enzymatic starch esterification reactions and the results suggested that no other effect other than faster and more efficient heating is imposed on the reaction (Lewandowicz *et al.*, 1997; Lewandowicz *et al.*, 2000; Xing *et al.*, 2006). Rajan and Abraham also carried out thermostability tests for the lipolase (Rajan *et al.*, 2006) under microwave radiation and concluded that the enzyme retains its activity for a short initial period of time. The degree of substitution was calculated by saponifying the attached fatty acid with a known excess of NaOH and backtitrating to the initial pH with a standard HCl solution, according to the method proposed by Miladinov and Hanna (Miladinov & Hanna, 1999) for starch acetate DS measurement. The esterification was also confirmed by Fourier transfer infra-red spectroscopy, showing the shift of the carbonyl of carboxylic acid group to the carbonyl of ester group. Higher degrees of substitution were reported for the microwave radiation assisted reactions (DS=0.98-1.55), while the liquid state reaction was successful for the Amano lipase PS (DS=1.1), but not for the lipolase (DS=0.07-0.08). The semi-solid state reaction was calculated to have an intermediate degree of substitution (0.43).

4.1.4 AKD-starch esters

Alkyl and alkenyl ketene dimers are commercially important fatty acid derivatives used as sizing agents in the paper industry, equipping cellulose fibre with water

repelling capabilities, due to their hydrophobic nature (Bottorff, 1994). Qiao *et al.* (Qiao *et al.*, 2006), investigated the possibility of esterifying these low-cost materials onto different types of starch through biocatalytic reactions. The enzymes used in this case were again commercially available lipases and proteases. The reaction systems were liquid with the starch dispersed in an organic solvent (DMSO, DMF, DMAc), while it is unclear if an aqueous phase is present. The DS was calculated from ^{1}H NMR spectra and the most efficient enzyme was lipase from *P. fluorescens*, while higher AKD concentrations, longer reaction times, higher temperatures and pH values resulted in greater degrees of substitution. The researchers also examined the non-enzymatic reaction and found it substantially lacking, as excesses of substrate, higher temperatures and harsh alkaline conditions are needed for the reaction to proceed, while the final product had a lower DS, compared to enzyme catalysed reactions.

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Chapter 1

Titration analysis of starch esters

1.1 Introduction

When starch has been esterified, whether by conventional chemical or enzymatic routes, product analysis is important. The analysis of esterified starch samples, through quantification of acyl content, has been approached in several different ways. Early research focused on quantification for esters of cellulose, but was later adopted for starch. The earliest techniques employed, initially for acetyl esters, always involved saponification of the acyl group (Whistler, 1945). The seemingly oldest and most common method, applied saponification in aqueous alkali. The technique proposed for cellulose acetate quantification by Genung and Mallatt (Genung & Mallatt, 1941), is actually based on an even earlier application and thus is referred to as the Eberstadt method (Eberstadt, 1909; Knoevenagel, 1914). The principle of the method is that if modified carbohydrate is treated with a known amount of alkali (e.g. NaOH), the ester bonds will be hydrolysed and acylate salt will form. If this solution is in turn titrated with a standard acid solution (e.g. HCl), the amount of alkali used for saponification can be calculated and consequently the acyl group substitution can be quantified.

In their original report, Genung and Mallatt (1941) did stress that this scheme presents several inherent drawbacks. The fact that heterogeneous conditions are required for the saponification makes the physical form of the solid ester a crucial variable. The diffusion of the reactants in and out of the solid particles, may cause under- or over-estimations of the acyl content. If this hurdle is overcome by dissolution in solvent, the method is then limited to soluble esters. This is not always the case especially when the solvent is mixed with large quantities of alkali. Polysaccharides tend to decompose in strong alkaline solution, producing acidic moieties, leading to overestimation of acyl content. The difficulty to produce standards and the difference in reaction conditions for simpler esters (e.g. glucose esters), make testing the method's accuracy extremely difficult. Finally the authors clearly state that this method is unreliable for esters of fatty acid, larger than butyric acid.

However, this quantitative analysis of starch acylates has been a popular method (Wurzburg, 1964) and is still widely employed (Chi *et al.*, 2008; Gao & Nishinari,

2004; Miladinov & Hanna, 1999, 2000; Rajan & Abraham, 2006; Rajan *et al.*, 2006; Rajan *et al.*, 2008; Saartrat *et al.*, 2005), without any reference to the difficulties described by the authors of the original report. In recent years and with the introduction of specialised high throughput titration tools, there has been no attempt to our knowledge, at a more insightful look at the parameters of this method. We attempted to perform this analytical approach, with an automatic titrator (Radiometer Analytical, TIM 854), which allows for precise readings and accurate titration of samples of small volume.

1.2 Materials and methods

1.2.1 Reagents

Wheat starch was a gift from BASF (Germany), while decanoic acid, sodium hydroxide and HCl 0.5 M (volumetric grade) were obtained from Sigma (UK).

1.2.2 Titration analysis (Miladinov & Hanna, 2000; Rajan & Abraham, 2006; Rajan *et al.*, 2006)

Titration analysis was performed in an automatic titrator (Radiometer Analytical, TIM 854). The substituted starch (300 mg) was treated with H_2O (3.6 mL) at 30^oC for one hour, followed by measuring the pH, and further treating with 1.4 mL 1.0 M NaOH at 50^oC for 48 hours (Miladinov & Hanna, 1999). The solution was then titrated with a volumetric HCl 0.5 M solution until the pH reached a value of approximately 1.5, so the full titration curve could be observed. The literature method would suggest DS is calculated from the amount of NaOH consumed during the saponification, after the excess has been titrated back to the pH value measured prior to NaOH addition.

1.3 Results and discussion

1.3.1 Technique overview

The procedure used (Miladinov and Hanna 1999) involved swelling the substituted starch in water, then measuring the pH, and saponifying with NaOH. The solution is then titrated with a volumetric HCl solution back down to the measured pH. In this way all the excess NaOH is titrated and the actual amount used for saponification can be calculated. This in turn allows the calculation of acyl groups substituted in the modified starch sample used. This can then be used to calculate the degree of substitution (DS):

$$DS = \frac{N_{FA} \times MW_{AGU}}{W - N_{FA}(MW_{FA} - MW_{H_2O})}$$

Equation 1.1 Equation for determination of DS with titration analysis. N_{FA}: moles of titrated fatty acid, W: weight of sample (g), MW_{FA}: molecular weight of the fatty acid, MW_{AGU}: molecular weight of anhydroglucose unit (162), MW_{H2O}: molecular weight of water (18)

In order to test out the method it was deemed necessary to first run control titrations. The first control was the titration of a known amount of NaOH 1.0 M (not standardised) solution, with the volumetric HCl 0.5 M solution.

In Figure 1.1 it is clear that the behaviour is very close to the expected, the small difference between the experimental and theoretical curve can be attributed to the fact that the NaOH solution is not volumetric and thus exactly 1.0 M, but as can be seen from the results, of a slightly smaller value.

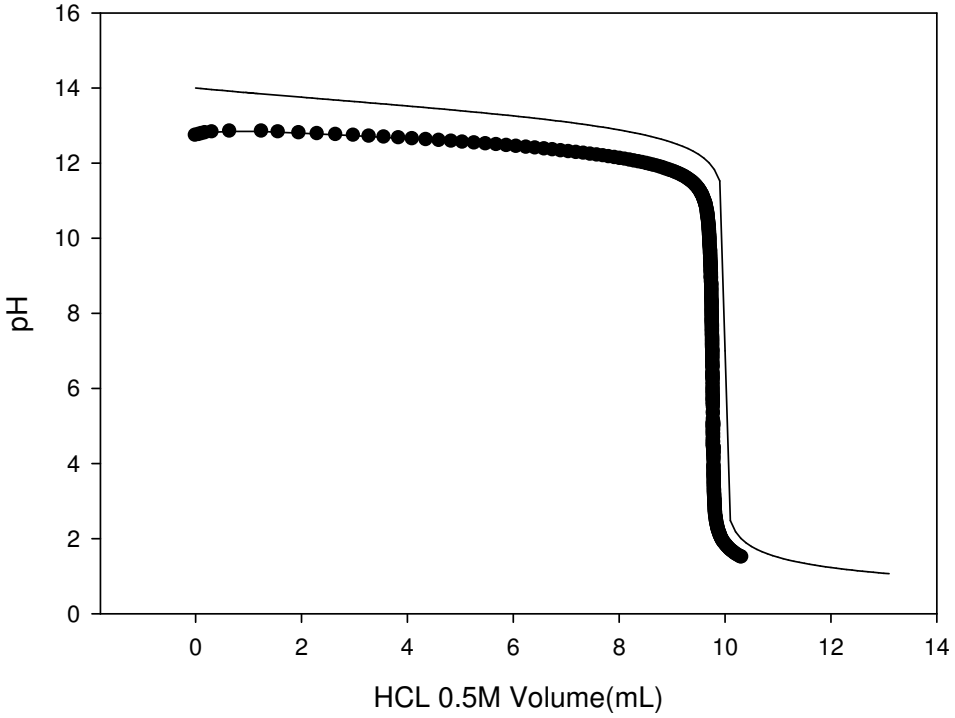

Figure 1.1 Titration curve for 5 mL NaOH 1.0 M solution, (—) Theoretical curve, (●) Experimental curve

1.3.2 Titration of fatty acid soap

The next step was to check the pH at which the sodium decanoate formed would titrate, in order to make sure that it is lower than the pH values obtained for the sample solutions. In this way when titrating back to that value with HCl, no amount of HCl will be used up for the sodium decanoate, thus giving us a larger reading than the correct one. A solution of sodium decanoate was made up by simply mixing NaOH 1.0 M and decanoic acid. This was then titrated with the volumetric HCl 0.5 M solution.

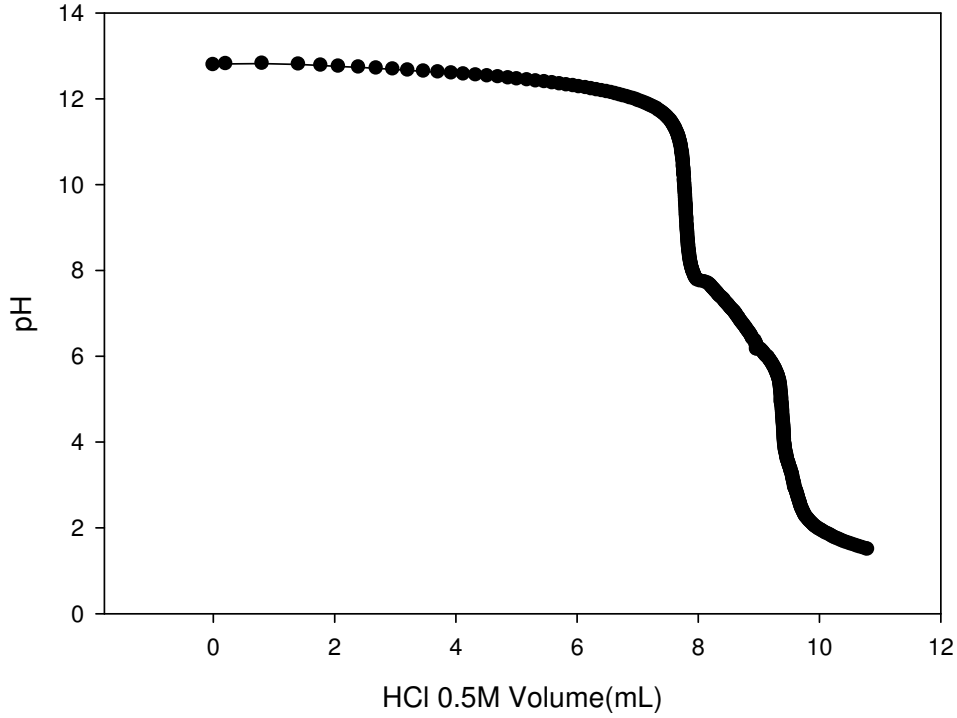

Figure 1.2 Titration curve for sodium decanoate

As can be seen in Fig. 1.2 the sodium decanoate begins to titrate at a pH of around 8. The titration occurs well above the true pKa value (approx. 4.8), presumably because the neutralised fatty acid comes out of solution, pulling the equilibrium over. This means that the starch ester solution after treatment with water should have a pH value of more than 8.0, in order for the results not to be affected by this secondary titration. If the actual starch pH is lower it can be corrected to a higher one possibly with addition of NaOH, before the pH measurement step. Note that the pH should also not be above 10, or the residual NaOH will not be completely titrated. Neither of these limits are noted in the published method.

1.3.3 Test of model system

Other necessary controls were a native starch sample and a native starch and decanoic acid mixture sample treated with the aforementioned method. Two samples each containing 300 mg of starch and 3.6 mL of H_2O were mixed and to one 50 mg of decanoic acid were added. These were incubated while shaken at 30^{o}C for 1 hr, after which 1.4 mL of NaOH 1.0 M were added to each sample vial and they were further incubated for 48 hr at 50 ^{o}C. After this treatment the two samples were titrated with the volumetric HCl 0.5 M solution. It should be pointed out that the automatic titrator stirs the solution at 1100 rpm and the titration speed was set to the lowest rate (0.001 mL s^{-1}). This was aimed at allowing the solution to homogenise as much as possible between readings so there are no local areas of higher or lower pH, as the sample mixture was quite viscous.

The titration curves in Figure 1.3 are a typical example of those obtained from the above experiment. The sample containing native starch might be expected not to have a different curve to the standard NaOH 1.0 M titration, but actually there is an important deviation. There seems to be a generation of acid from the starch treatment, which neutralizes some of the sodium hydroxide. It should be noted that the viscosity of the solution is high, making it difficult to homogenise the solution during titration. This may cause localised higher or lower pH values, resulting in inaccurate titration curves. The drop of pH at lower volumes of added acid for the native starch curve, could also be caused to a certain extent by partial oxidation which generates small carboxylic acids, such as ethanoic and lactic acid, during the alkaline treatment of starch (Genung & Mallatt, 1941). This means that it is necessary to take into account this contribution when quantifying for a modified product. From these results it is clearly necessary to always use a titration of the native unmodified starch, as a blank. This blank is only mentioned in few early publications (Wurzburg, 1964). However, these starch blanks were found not to be very reproducible, nor to vary proportionally to the amount of starch used. Together with the fact that the starch blank can be a very large correction, this makes the method not reliable.

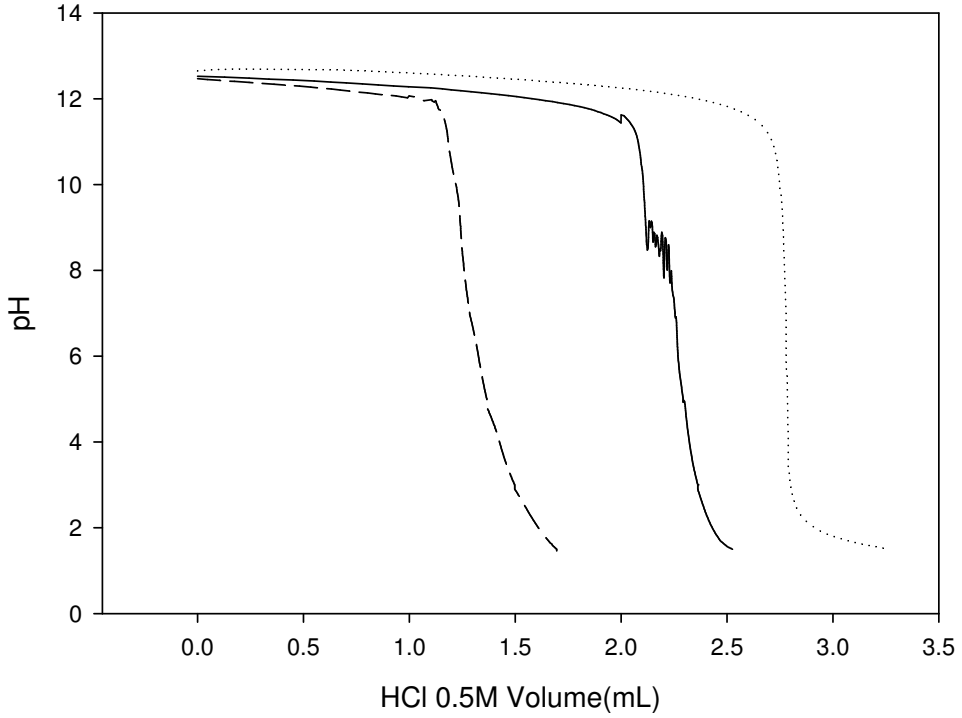

Figure 1.3 (⋯) 1.4 mL of NaOH 1.0 M, (—) Starch only standard titration curve, (– – –) Starch and decanoic acid titration curve (experimental points are very close to each other, so for clarity they are shown as lines)

Another complication of this method appears in the starch and decanoic acid mixture titration curve. As seen above, the titration of sodium decanoate is expected at a pH of around 8 to 6. However after saponification of the acid in a mixture with starch, the titration can not be observed (Figure 1.3). The curve does show a clear consumption of NaOH, attributable to neutralisation of the decanoic acid. But even though the initial neutralisation is visible in the curve, the subsequent titration of sodium decanoate can not be recorded. It was also observed that the pH of the starch mixture, prior to NaOH addition, had values close to 6. If the back-titration step is carried out to this pH, it would be expected that some sodium decanoate would also be titrated, thus adding to the error in the measured DS.

1.4 Conclusions

When investigating the possibility of using aqueous alkaline saponification and titration of fatty acid starch esters, as a method of quantification of the DS, a number of flaws arose. The heterogeneous nature of the titration mixture and the high viscosities limit the reproducibility and accuracy of pH measurement and analysis. Starch blanks revealed neutralisation of NaOH, potentially due to generation of acidity from the polysaccharide during alkaline treatment. Insolubility of longer chain fatty acids in aqueous conditions, results in titration of their soap at higher pH values than expected. If this is not taken into consideration, the required back titration to reach the initial starch mixture pH (prior to NaOH treatment), may overlap with areas of neutralised fatty acid titration. Some of these issues have been raised in original reports of this method (Genung & Mallatt, 1941), however it has subsequently been employed without apparent regard for these setbacks and used for esters of longer chain fatty acids, where the method is inherently flawed. These experimental observations led to the conclusion that this method is unreliable, at least for starch esters of medium chains.

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Chapter 2

GC/FID analysis of starch esters

2.1 Introduction

Another method, much less documented in recent literature (Tomasik & Schilling, 2004), for the quantification of acetylated carbohydrates, involves methanolysis in anhydrous methanol, thus forming methyl acetate (Whistler & Jeanes, 1943). Instead of direct saponification of the starch esters, like in the previously described method (Genung & Mallatt, 1941), an initial transesterification of the acetyl to methanol takes place, followed by saponification and back-titration but this time of the methyl ester. The methyl acetate formation step aims to resolve some of the setbacks of the older technique, since the solution to be saponified is solely purified methyl ester (Whistler, 1945).

In the case of starch esters of longer chain fatty acids, the methyl esters may be harder to distil, while the extra methyl ester formation step adds to the complexity of the procedure and decreases accuracy. This may be why said method is rarely cited in modern literature. However fatty acid methyl esters (FAMEs) can be easily detectable with great accuracy on a gas chromatography system, with a common detector such as a flame ionization detector (Eder, 1995). No reports for GC analysis of methyl esters from acylated starch could be found. There has been an early report (article in german) on GC analysis of methyl acetate from acetylated cellulose (Wandel and Tengler, 1966), however the article could not be retrieved and details are not known. We therefore investigated GC detection of FAME as an alternative to the titration method described above.

2.2 Materials and methods

2.2.1 Reagents

Methanol was of anhydrous grade and was purchased in a Sureseal® container, as was sodium methoxide in methanol 0.5 M (Sigma, UK). Decanoic acid methyl ester standard was also obtained from Sigma (UK).

2.2.2 Transesterification of starch esters

A suitable method is aliquoting a small starch sample (5-30 mg of polysaccharide as dry solid or dissolved in 0.5 mL DMSO) and adding 1 mL of sodium methoxide 0.07 M in methanol solution and a known amount (10 μL of a 100 mg mL^{-1} solution in n-heptane) of internal standard (n-tetradecane was suitable in the case of decanoic acid). The reaction is run in 10 mL quickfit test tubes, with quickfit air condensers attached, while incubated in a heating block, or an air incubator if shaking is required (30-60^{o}C), for a suitable amount of time.

2.2.3 GC/FID analysis of fatty acid methyl esters

After transesterification, 1 mL of water and 1 mL of n-heptane are added to the reaction. The mixture is shaken for 1 min and left to settle. The top organic phase contains the methyl ester and can be removed and ran through the GC-FID. The GC system used is a Perkin-Elmer Autosystem XL with a CP Simdist capillary column. The oven is set at 120^{o}C, the injector at 130^{o}C and the detector at 150^{o}C.

2.2.4 Methyl ester standard

The retention time of decanoic acid methyl ester was confirmed with standard produced by acid-catalysed esterification of the free fatty acid with methanol. A 1-2% solution of concentrated sulphuric acid in methanol was mixed with fatty acid and refluxed for 2 hr. After this water (1 mL per mL methanol) and heptane (2 mL per mL methanol) were added and mixed thoroughly and the phases are left to separate. The methyl ester was extracted into the top phase, which can be injected into the GC. The detector response is known to be proportional to the number of carbons active towards a response (all carbons excluding carbonyl carbon for FAMEs) (Ackman & Sipos, 1964). The use of theoretical relative response factors (RRF) based on this observation has been confirmed as accurate (Bannon et al., 1986). Corrections to the theoretical RRF for differences in chain length and alkyl unsaturation are usually smaller than other expected errors in the procedure (Ackman & Sipos, 1964; Bannon et al., 1985). In the case of decanoic acid, retention time and RRF were confirmed with decanoic acid methyl ester standard purchased from commercial source (Sigma).

2.3 Results and discussion

2.3.1 Triglyceride test

This method was tested out for tricaprin, the triglyceride of decanoic acid. This test allowed for optimisation of the GC analysis, for speed, accuracy and reproducibility. The peaks obtained were at 0.18 min for the solvent (n-heptane), 0.41 min for the decanoic acid methyl ester (DAME) and 0.56 min for the internal standard. Calculations showed that the reaction worked with 100% conversion of the triglyceride with no visible by-product peaks.

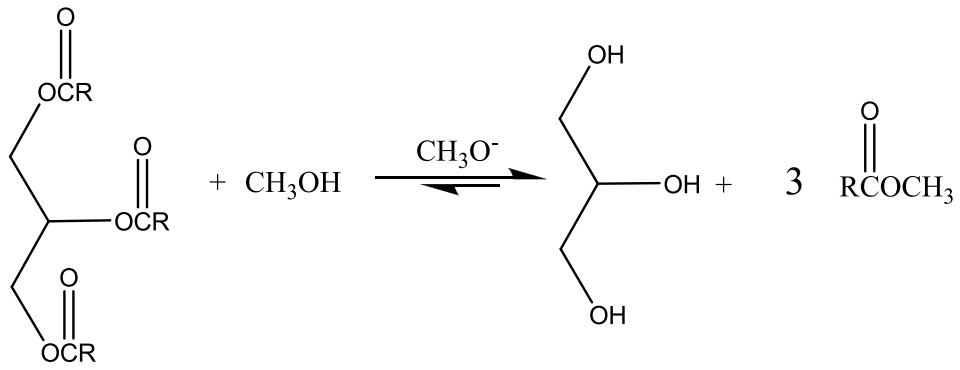

Figure 2.1 Alkaline methanolysis of tricaprin yielding glycerol and decanoic acid methyl ester

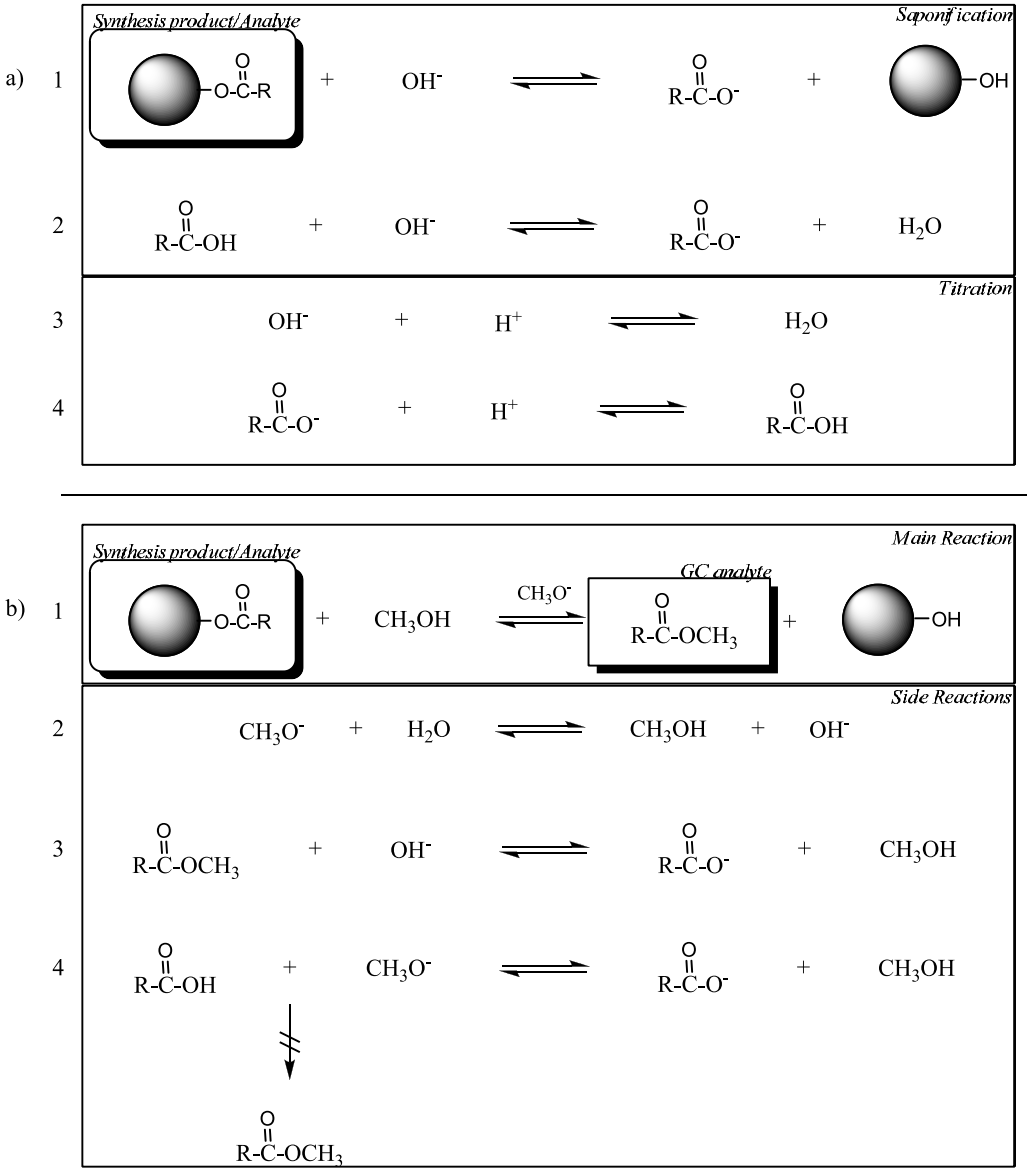

Figure 2.2 Comparison of reaction schemes in a) Titration Analysis: 1 Hydrolysis of starch ester (circle denotes starch), 2 Saponification of acid, 3 Titration of residual hydroxide ions, 4 Titration of saponified acid and b) Transesterification/GC Analysis: 1 Methanolysis of starch ester and formation of methyl ester, 2 Hydrolysis of methoxide 3 Hydrolysis of methyl ester, 4 Deprotonation of acid to form acyl ion, no methyl ester synthesis.

2.3.2 Starch ester transesterification

The method was applied to samples of starch esters from reactions and control reactions carried out (see chapter 4). Approximately 20 mg of product were used in each case. The scheme's precision as an analytical technique, was tested by analysis of identical samples for the same as well as modified transesterification conditions. But when samples of the same weight taken from a reaction batch were analysed, the result was not reproducible. Furthermore no logical trend could be distinguished for different systems tested with different concentrations of sodium methoxide, different reflux times and different sample weights. In all cases the results obtained for solid samples aliquoted from a single batch of starch ester, were erratic. However in the case of reaction systems carried out in small scale, where most of the product obtained was used for analysis, the results were reasonably reproducible for the aforementioned conditions. This indicates increased heterogeneity of the solid starch reaction product, either due to uneven distribution of acylated sites or differences in physical condition -causing differences in the heterogeneous alkaline transesterification kinetics- throughout the solid batch of starch.

Sample dissolution in DMSO was tested as a way to homogenise the batch sample and divide it into identical aliquots. The DMSO did not seem to cause any problems with the transesterification mixtures and reproducible results were obtained for samples from different wheat and maize starch larger scale reactions, as shown in Table 2.1.

Reaction ("semi-solid system")	Precision (standard deviation of triplicate DS analysis)
Maize starch	0.004±0.0016
Wheat starch	0.004±0.0008

Table 2.1 Transesterification/GC results after DMSO dissolution

2.3.3 Transesterification parameters

The effect of different transesterification parameters such as incubation time and temperature were also investigated. Initial results, for boiling methanol, showed that there is a maximum for the transesterification yield at around 30 min. At 45 min the DAME quantity starts to decrease, suggesting that degradation of the FAME, from a hydrolysis side-reaction due to water presence, is important. This means that it is necessary to take steps to keep the transesterification system as dry as possible, by use of special grade reactants or further drying the starch samples.

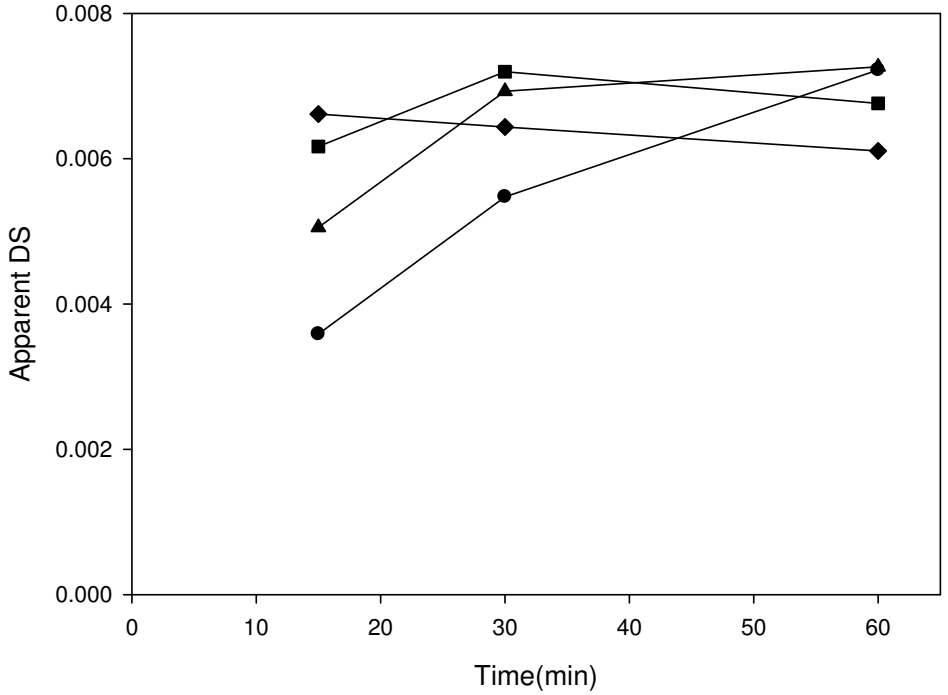

Figure 2.3 Transesterification/GC results for different incubation times at 30°C (●), 50°C (▲), 80°C (■) and 105°C (♦), while stirred at 30°C and 50°C

Tests were run for a range of temperatures and for different incubation times (Figure 2.3). The results showed that at high temperatures the hydrolysis reaction of the methyl ester caused the DAME peak to decrease after a specific time. The optimum transesterification conditions were found to be at 50°C for 60 min. At this temperature there is little hydrolysis and the DS reaches a plateaux after one hour. It

was also concluded that stirring the solution made the reaction proceed faster, which means that diffusion into the solid phase is important.

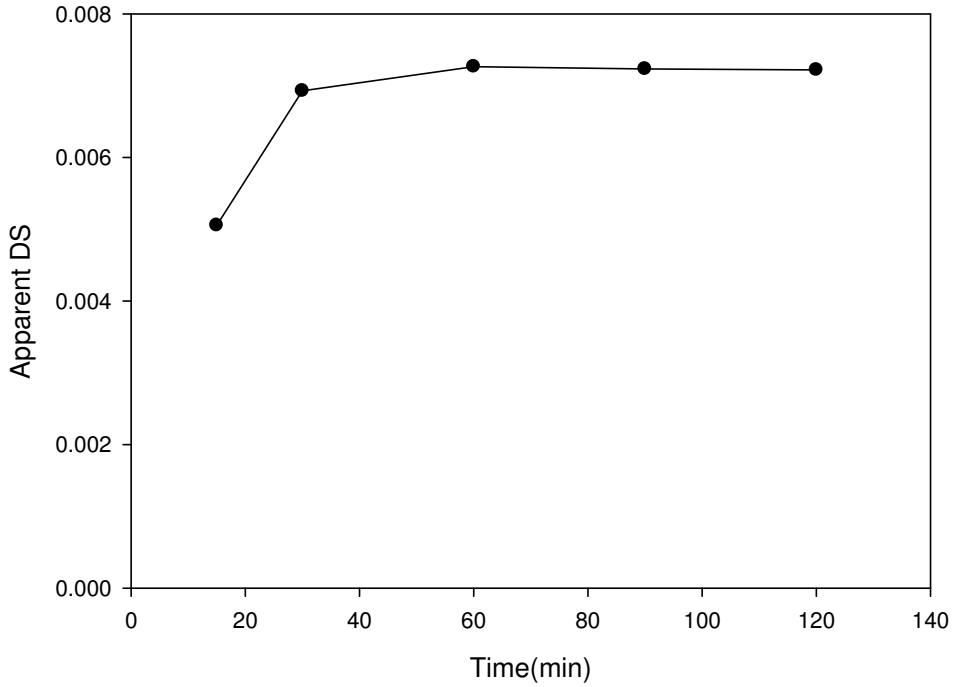

Figure 2.4 Transesterification/GC results for incubation at 50°C over time

2.4 Conclusions

The possibility of combining the transesterification of starch esters with CG/FID analysis of the resulting methyl esters, was investigated for starch esters of decanoic acid. Gas chromatography was found to give accurate and precise results for the fatty acid methyl esters prepared. The transesterification step was found to depend on the starch ester's physical properties, the purity of the solvent and the reaction conditions. Reproducible results were observed when:

-The solvents used were anhydrous, in order to suppress side-hydrolysis of the produced ester.

-The starch sample was homogenised by dissolution in DMSO.

-The reaction was incubated at 50^{o}C for 1 hour with mixing.

In conclusion the combination of alkaline transesterification and gas chromatographic analysis of the resulting methyl esters was found to be an accurate and reproducible analytical scheme for measuring the DS of starch ester of middle to long chain fatty acids. The technique could be employed for short chain fatty acids, but the volatility of the resulting methyl esters will require special treatment for the chromatographic analysis.

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Chapter 3

NMR analysis of starch esters

3.1 Introduction

The use of nuclear magnetic resonance as a potential tool for direct analysis of modified starches has been of great interest to researchers, due to the availability of instrumentation, as well as the potential of detailed product characterisation. Of course the use of NMR for analysis of polymers presents problems, mainly due to the need for a dissolved sample in concentrations that are not always achievable with polymers and the low resolution of obtained spectra.

Laignel *et al.* (Laignel *et al.*, 1997) studied the spectrum of acetylated starches and argued that NMR could be a potentially valuable tool for the analysis and characterisation of highly substituted starches. However they found problems in the detection of certain acetylation sites, which they attributed to the presence of non solvated regions of polysaccharide material. Heins *et al.* (Heins *et al.*, 1998) published a comparison of acetylated and hydroxyethylated starch, using ^{13}C NMR amongst other analytical techniques.

Elomaa and co-workers (Elomaa *et al.*, 2004), investigated the potential quantitative analysis of acetylated starches with ^{1}H-NMR and found the method to be problematic. The hygroscopicity and high viscosity of samples dissolved in DMSO-d_6, resulted in poor spectra. They found that a DMSO-chloroform mixture (1:1), much improved the observed spectra, however, this cannot be employed for low substitutions, because those starch esters are insoluble in such solvents. Also for lower DS values the backbone signals were found to get broader and overall accuracy of quantification dropped. Again, this is probably an effect of decreased solubility for lower DS values. They concluded that NMR should be avoided for quantification of samples with DS values below 2.0.

Despite these reports, NMR spectroscopy has been used in many cases to quantify DS for starch esters, in recent years, usually in conjunction with other methods (Aburto *et al.*, 1999; Dicke, 2004; Shogren, 2008; Shogren, 2003). It is used in publications with reports of enzymatic acylation, even where substitution is low (Chakraborty *et al.*, 2005; Qiao *et al.*, 2006). However authors do not mention limitations or discuss issues observed in previously published work. The DS values obtained by Chakraborty and coworkers may be sufficiently high (0.2-0.9) to give

relatively safe estimations by NMR (though not according to the more pessimistic estimates for DS>2.0 of Elomaa *et al.*) , however in the case of Qiao *et al.* the values obtained seem particularly low for confident quantification.

We attempted to employ NMR spectroscopy to investigate the possibility of DS quantification of starch esters prepared by lipase catalysed acylation with decanoic acid (chapter 4).

3.2 Materials and methods

3.2.1 Reagents

DMSO-d_6 and methanol-d_4 were purchased from Sigma (UK).

3.2.2 NMR analysis

^{1}H and 2D-COSY experiments were conducted at 50^{o}C on an AVANCE/DRX500 spectrometer at 500 MHz. The starch samples were dissolved at 50^{o}C in DMSO-d_6 or DMSO-d_6 : methanol-d_4 (4:1) at a concentration of 10 mg mL^{-1}. The samples were incubated at 50^{o}C for 5 min in the spectrometer, prior to analysis. COSY experiments were conducted with the default Bruker Biospin gradient COSY programme (cosygpqf, first pulse 90 degrees, second pulse 20-90 degrees, 1.8 s relaxation time, spectral width of 3654 Hz in both dimensions).

3.3 Results and discussion

3.3.1 ^{1}H-NMR of starch decanoate, ethanol precipitated

We attempted to analyse the product of an enzymatic starch acylation by NMR and compared the spectrum with that of unmodified native starch (Figure 3.1). With the aid of a COSY experiment (Figure 3.2), we successfully assigned the peaks corresponding to carbohydrate protons and also found three extra significant peaks, appearing exclusively in the modified starch spectrum.

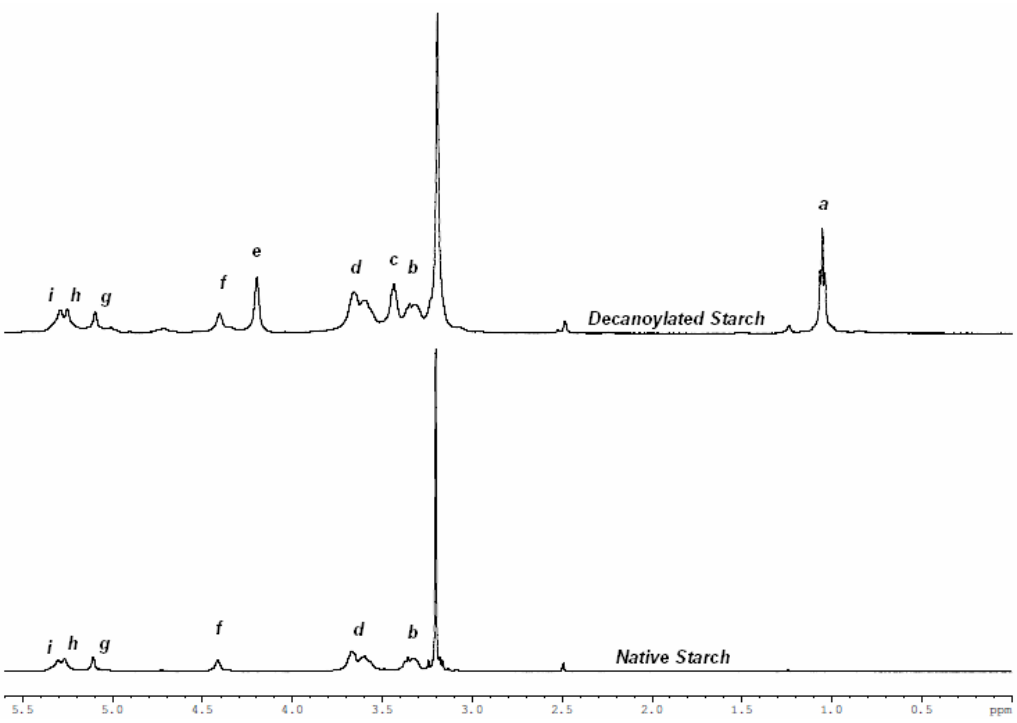

Figure 3.1 NMR Spectrum for starch decanoic acid ester and unmodified native starch. The large peak at 3.2 ppm can be attributed to water from the starch sample.

The upfield peak was at a shift that has been attributed to protons in the fatty acid chain in previous published work on starch fatty acid esters (Bruno *et al.*, 1995; Chakraborty *et al.*, 2005; Qiao *et al.*, 2006). A scan of the same sample after addition of deuterated methanol verified our peak assignments for the starch protons, as the hydroxyl proton peaks disappeared, but also resulted in the loss of one of the non-carbohydrate peaks (Figure 3.3).

Figure 3.1 shows the NMR spectrum for decanoylated starch ester. Peaks b (3.322-3.357 ppm), d (3.601-3.668 ppm) and g (5.108 ppm) can be assigned to the CH protons from the C4/C2, C5/C3/C6 and C1 anhydroglucose carbons respectively. Peaks f (4.415 ppm), h (5.264 ppm) and i (5.305 ppm) are due to protons from the hydroxyl groups of C6, C3 and C2 carbons respectively. Finally peaks a (1.006-1.072 ppm), c (3.446 ppm) and e (4.207 ppm) are the extra peaks that are not recorded on the native starch spectrum. As mentioned above, although the peak cluster a is where one would expect a signal from decanoyl protons, peaks in all these regions were recorded for ethanol, which was used to precipitate and wash the reaction product.

3.3.2 Identification of residual precipitant

The disappearance of one of the unidentified peaks (e in Figure 3.1) made us suspect the possibility of residual ethanol from the washing stages of the reaction. Our suspicions were verified by measurement of the spectrum of ethanol in the same DMSO-d_6 medium, showing perfect agreement of chemical shifts (Figure 3.4).

3.3.3 ^{1}H-NMR of starch decanoate, acetone precipitated

We further analyzed a sample of acylated starch precipitated with acetone instead of ethanol (Figure 3.5). The peaks attributed to ethanol were indeed now missing, with instead a large peak appearing at 2.08 ppm, as expected for acetone protons. Small low-shift peaks were revealed, which might be attributable to methyl and methylene protons in the decanoyl chains. Their low intensity made accurate quantitation difficult, but estimated DS values were about twice those from the GC method. However, fatty acid non-covalently attached to the carbohydrate would also contribute to these peaks. No ^{1}H-NMR peaks were distinguished that could be assigned exclusively to covalently (or non-covalently) bound fatty acid. Hence we conclude that this NMR approach is not a reliable method for estimation of DS in the low range encountered with our samples. It may well be accurate for much higher DS starch derivatives, especially when purified (as previously mentioned Elomaa *et al.* (2004) suggest that the method should be used for DS > 2).

3.4 Conclusions

Through ^{1}H-NMR analysis of decanoic acid starch esters (chapter 4), it was concluded that it is possible to obtain spectra with identifiable peaks. Due, however, to the low DS of the esters produced, the difficulty in distinguishing the contribution of residual non-covalently bound fatty acid and the variability of results depending on the sample work-up and history, it was concluded - in agreement with some literature (Elomaa et al., 2004) - that ^{1}H-NMR spectroscopy cannot be used for accurate measurement of the DS in starch esters, for low substitutions.

3.5 References

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3.6 Appendix: Supplementary NMR spectra

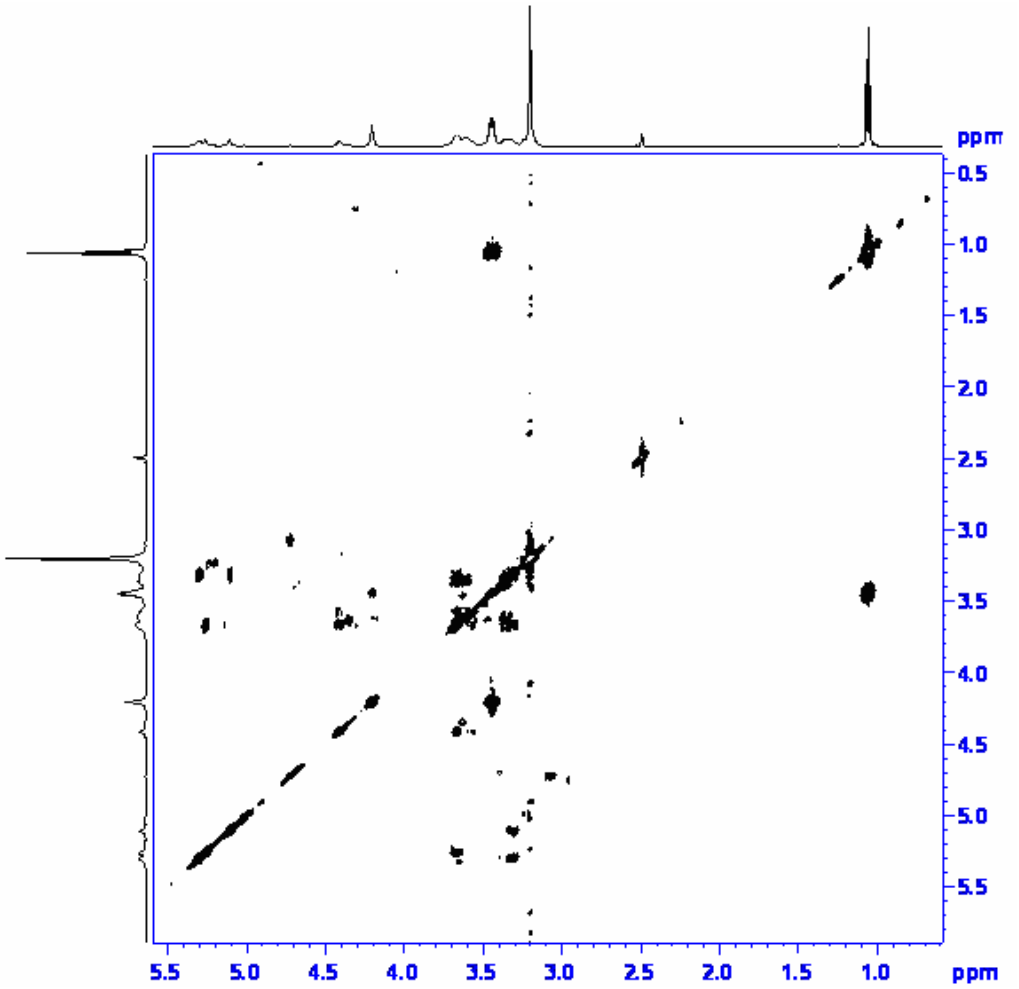

Figure 3.2 COSY spectrum for starch decanoate (figure 3.1)

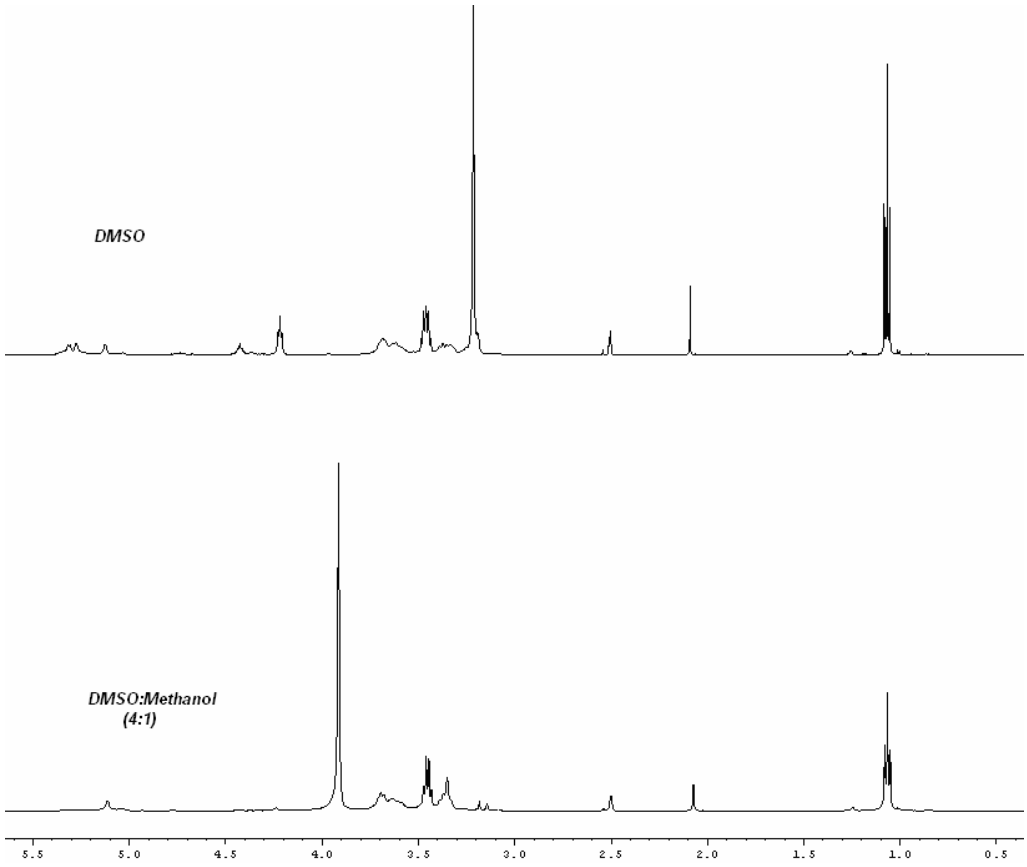

Figure 3.3 Spectra of decanoylated starch obtained for different deuterated solvents, when CD_3OD is added to the solvent the peaks attributed to hydroxyl protons are suppressed.

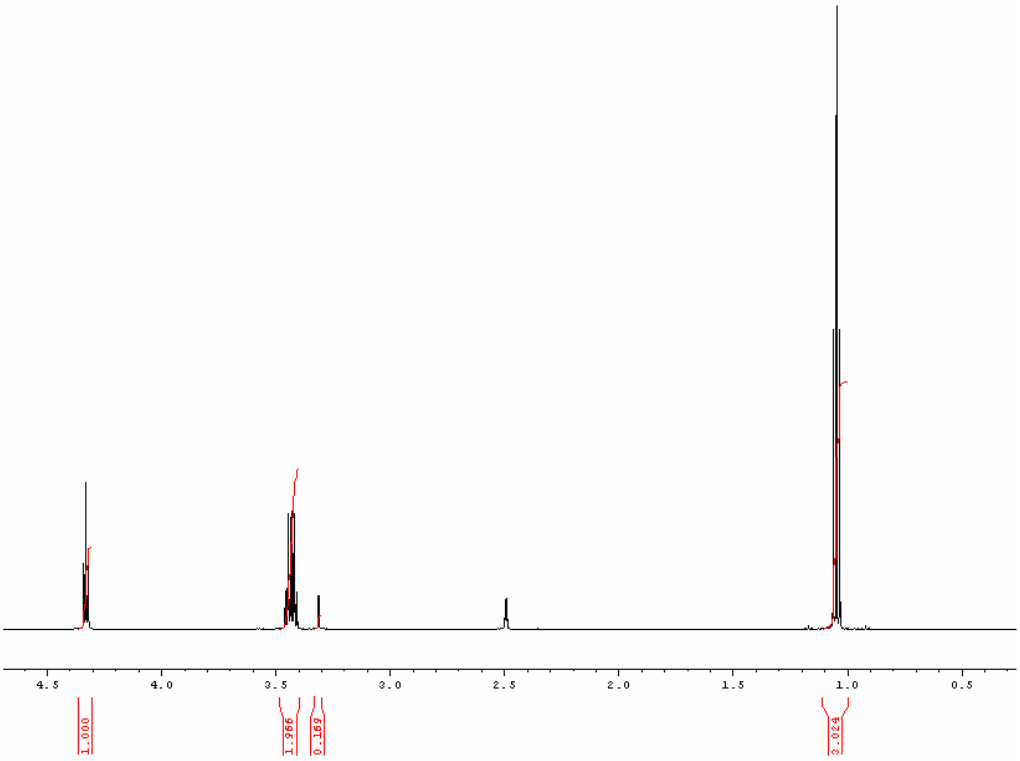

Figure 3.4 Spectrum of ethanol in DMSO-d_6

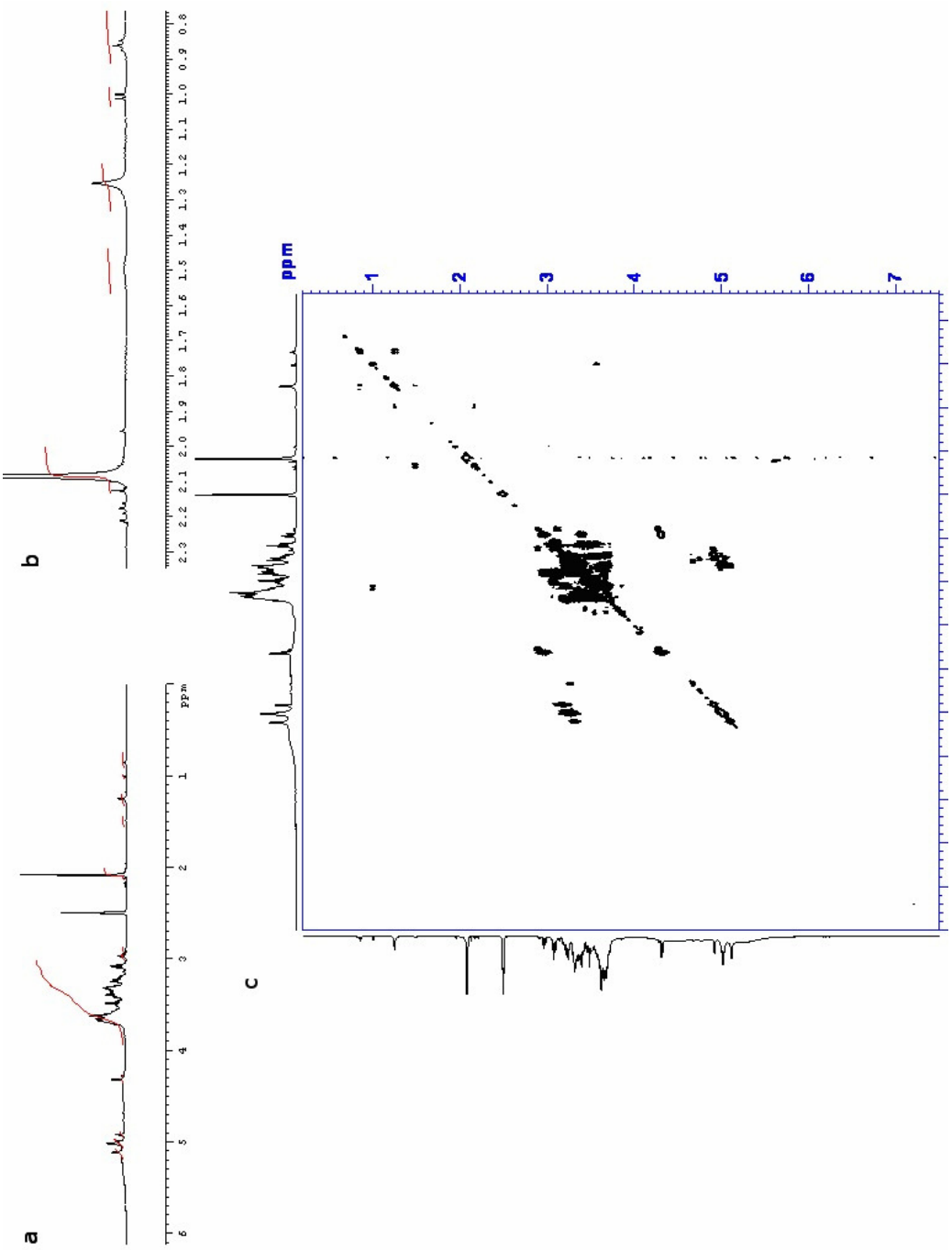

Figure 3.5 NMR spectra for acetone precipitated starch decanoate, ^{1}H-NMR spectrum (a), magnification of upfield peaks (b) and COSY spectrum (c).

Chapter 4

Enzymatic acylation of gelatinised starch

4.1 Introduction

One of the main problems when attempting to acylate starch with the aid of a biocatalyst is the heterogeneous nature of the system. The hydrophobic nature of the acyl donor (in the case of medium/long chain fatty acids), coupled with the virtual insolubility of starch in most solvents, means that one phase where acyl donor, nucleophile and catalyst will co-exist in solution is unlikely.

It seems that the main target is to successfully bring into contact the three ingredients, in a manner sufficient for biotransformation to take place. It is well known that an aqueous dispersion of starch can be turned into a sol or gel, by heating at high temperatures, through the process of gelatinisation. This does not result in a true solution, since the macromolecules continue to interact non-covalently, especially when cooled, however, it does produce a continuous aqueous phase of polysaccharide. Especially in the case of high carbohydrate concentrations the resulting phase is very viscous and gel-like (depending on the starch source). It may be possible to disperse fatty acid throughout this system, creating sufficient interface for reaction to proceed. This could be aided by addition of surfactant.

The hydrolytic action of hydrolases is thermodynamically favoured when acting in water. This is definitely the case for dilute aqueous solutions, which is the norm in enzymology. However, it has been reported that when enzymes act on solid surfaces, under aqueous conditions, the preference of hydrophobic groups on dissolved substrates for a less hydrated environment (such as that of the solid) may lead to a shift of equilibrium towards synthesis (Halling, 2006). In the case of gelatinised starch the way in which water would take part in the reaction is also unclear, since water molecules may be considered as bound between the interacting polysaccharide chains or even interacting non-covalently with the carbohydrate themselves.

Here the acylation of gelatinised starch, catalysed by a lipase, is tested. Lipases are considered the workhorse enzyme for synthetic reactions and an obvious choice when choosing a catalyst to start with. Fatty acids are a natural substrate for lipases and decanoic acid in particular is liquid at relatively low temperatures (approx. 40^{o}C), where the lipase catalysed reaction can be performed.

Similar systems have been reported in literature (Rajan & Abraham, 2006; Rajan *et al.*, 2006; Rajan *et al.*, 2008), though specific reaction conditions are unclear and the analytical technique used for quantitation (saponification/titration), as we have seen (chapter 1), leads to erroneous results. Therefore the potential of this mixture as a reaction system for the synthesis of starch ester with decanoic acid was investigated.

4.2 Materials and methods

4.2.1 Reagents

Tapioca starch, Amioca Powder TF (high amylopectin), Hylon VII (high amylose) were gifts from National Starch (Germany). Maize and wheat starch were gifts from BASF (Germany). Potato amylose, lipase from *Thermomyces lanuginosus* (lipolase, L0777), Triton X-100, decanoic acid, vinyl decanoate, sodium phosphate (monobasic and dibasic) were purchased from Sigma (UK).

4.2.2 Concentrated Aqueous System

An appropriate amount of sodium phosphate buffer (100 mM, pH=7.0) and 2 g of dry starch (dried overnight at 60^{o}C), were mixed in a 50 mL round-bottom flask. The starch was gelatinised by heating in a water bath at 90^{o}C, for approximately one hour, while mixed by overhead stirring (Heidolph overhead stirrer, anchor paddle, 50 rpm) and subsequently cooled to the reaction temperature. To the cooled mixture, 0.5 mL (50 kU, Sigma assay) of lipolase solution (lipase from *Thermomyces lanuginosus*) and 0.5 g of liquid decanoic acid (preheated at reaction temperature) were added per g of starch (0.46 mol decanoic acid per mol anhydroglucose). This reaction mixture was then mechanically agitated by overhead stirring, while heated, until the completion of the reaction. Control systems contained phosphate buffer instead of lipase solution.

The reaction mixture is then diluted to ten times its volume, with a water miscible organic solvent such as methanol, ethanol or acetone, causing the starch to precipitate (very polar organic solvents like DMSO do not act as precipitants). The starch is then isolated, by centrifugation (3,500 rpm, 5 min) and washed twice with

an equal volume of the precipitant. Finally, the product is dried in a desiccator under vacuum for a few hours and overnight in a fan assisted convection oven at a low temperature (40-50^{o}C).

Smaller scale reactions were employed typically in 2 mL screw-top microcentrifuge tubes, with 50-100 mg of starch and the same ratio of ingredients. Gelatinisation is performed on a heating block, while the reaction is incubated in an air-incubator. Constant mechanical mixing was performed on a Vortex Disruptor, with the aid of glass beads (0.05-0.1 mm diameter).

Errors were calculated as standard deviations of experiments performed in triplicate.

4.2.3 Anthrone analysis for sugar quantification

When reactions were run on smaller scale, there was difficulty in separating the starch esters from the glass beads that were required for adequate mixing. Though they could be removed after DMSO dissolution of starch, prior to GC/FID analysis, this meant that the sugar ester could not be weighed. For this reason quantification of carbohydrate content in the DMSO sample solution was necessary. This was done with the aid of an anthrone assay. In this method, the carbohydrate is initially dehydrated with a strong acid to yield furfurals, which in turn form a coloured complex with anthrone. In the case of glucose and its polymers, 5-hydroxymethylfurfural is formed as the intermediate compound (Figure 4.1).

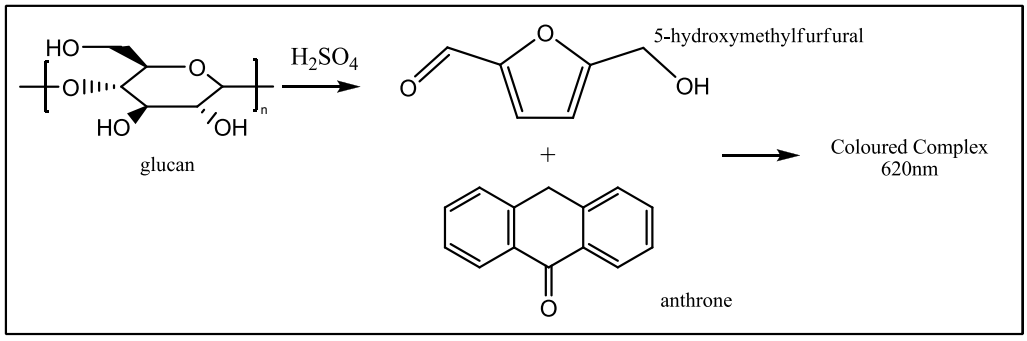

Figure 4.1 Glucose polymers are hydrolysed and dehydrated yielding 5-hydroxymethylfurfural, which will form a coloured complex with anthrone.

The reagent is a 10mM solution of anthrone in sulfuric acid (1.954 g L^{-1}). Typically, 100 mL batches were prepared freshly but no less than 4 hr prior to the experiment and stored in a fridge and away from the light.

A starch sample in DMSO of approximately 10 g L^{-1} would be diluted 100 times with DMSO and aliquoted into an amount of deionised water, to give a final concentration in the range of 1 mg L^{-1} (i.e. diluted 10000 times). This solution would then be mixed with the anthrone reagent at a ratio of 1 : 1.5 and incubated at 100^{o}C on a heating block for exactly 15 min, after which time the sample would be cooled down to room temperature in a refrigerator (approximately 10 min). The absorbance of the sample is measured on DU 800 Beckman-Coulter UV-Vis Spectrophotometer, at 620nm, where the solutions were found to present a maximum. Blanks contained no carbohydrate sample in the deionised water. The carbohydrate concentration was quantified from the measured absorbance with the aid of a calibration curve, prepared with glucose standards.

4.3 Results and discussion

4.3.1 System mixing

Systems using lipase-catalysed esterification of gelatinized starch with a low water concentration, surfactant and a free fatty acid have been described in literature, though reaction details are unclear (Rajan & Abraham, 2006; Rajan *et al.*, 2006). In our hands, the homogeneity and ability to be mixed of these systems is low. This is why strong mechanical agitation was necessary for the mixing of the reaction. We found that an overhead stirrer provided adequate mixing, for the ingredients to come in to contact and the reaction to proceed in a reproducible manner (Figure 4.2).

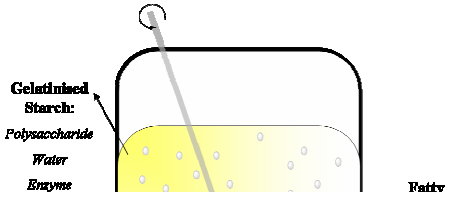

Figure 4.2 Representation of ingredient distribution in the concentrated gelatinised starch reaction mixture.

4.3.2 Importance of precipitant

Starch that has been dispersed or dissolved in polar solvents is often removed by precipitation with an organic solvent that is miscible with the original solvent, but less polar. Dilution with such a solvent causes a net drop in the polarity of the liquid phase, causing the starch to precipitate. In most cases absolute ethanol has been the precipitant of choice. However when ethanol was used in the lipase reaction described, it led to formation of small but detectable amounts of ethyl decanoate, possibly through enzyme catalysis. The amounts found (by GC analysis) corresponded to less than 0.0005 DS but it is recommended to avoid nucleophilic precipitants, such as ethanol, especially when low DS values are to be measured. Therefore the use of a acetone as a precipitant is recommended.

4.3.3 Starch type

The analysis of these enzymatic reaction systems with the GC method described earlier (chapter 2) showed clear peaks of methyl decanoate, whilst controls (no enzyme) gave no peaks. Quantification of these peaks showed that starch decanoate with a DS ranging from 0.002-0.009 was obtained (Figure 4.3). Such DS values are close to 0.01, which is approximately the DS for most starch acylates with desirable properties. Further acylation alters too much the physical characteristics of starch, due to the strong hydrophobic nature of the acyl groups (Jarowenko, 1986).

Tapioca shows high substitution which may be attributed to the fact that it is classed as a C-type polymorph (Tester *et al.*, 2004). This means that the amylopectinic fraction has a more open structure. Gelatinisation though, should have disturbed starch organisation at this level. Another point is that, in general, starches with higher amylopectinic fractions seem to result in higher DS values. This probably can be related to the behavior of the starch after gelatinisation, rather than the chemistry of the actual molecule itself, which likely influences the mixing of the system as well. Starches with a higher amylopectinic content result in a more continuous and sol-like phase when gelatinised. This mixture is clear and acts like a very viscous fluid, becoming more and more viscous for higher concentrations of starch (Figure 4.7a). During the enzymatic reaction it becomes cloudy when the fatty acids is dispersed throughout. On the other hand starches with higher amylose contents are opaque and

lumpier when gelatinised (Figure 4.7b). When mixed the gel breaks up into lumps that act like solids, while in the reaction mixture the fatty acid seems not to disperse homogeneously but rather coat the surface of these lumps. This effect may result in better mixing of the reaction ingredients for higher amylopectin ratios.

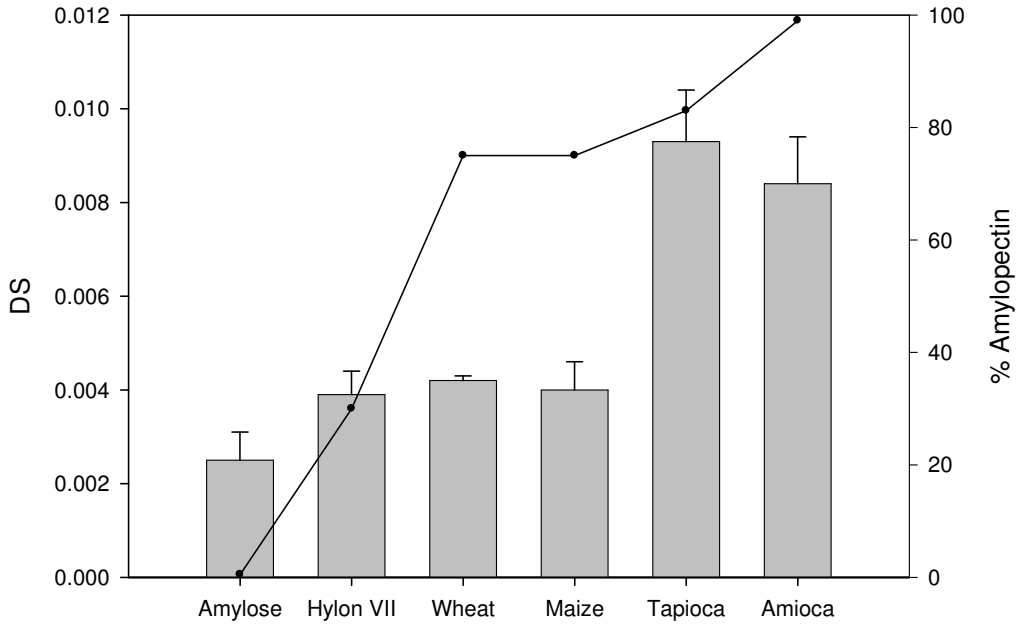

Figure 4.3 DS obtained for the enzymatic synthesis of decanoic acid esters (50°C, 60 min) , for different starch types (bar chart). The points indicate % amylopectin present in the different native starch substrates.

However it would be expected that from a molecular point view, if any one molecule was preferred by the enzyme, that would be the unbranched amylose for steric reasons. The slight drop in substitution for totally amylopectinic starches might be a result of this. Amylose is the molecule that reacts better, while amylopectin aids in the creation of the suitable conditions for reaction to take place. There may be a trade-off therefore, resulting in an optimum ratio of the two substituents.

4.3.4 Reaction progress with time

The reaction progress with time was monitored for the starch type that resulted in the higher DS (tapioca). From Figure 4.4 it is obvious that the reaction reaches a plateau

after one hour, though whether it is a true equilibrium can not be deduced from these data alone.

It is worth noting that a control where the enzymatic reaction mixture is analysed without incubation (yield for time zero), did not result in the generation of methyl ester. This control was run in case any methyl ester was generated from residual fatty acid, catalysed by residual lipase, with precipitant alcohols during the work-up and methanol during the analysis process (e.g. as a result of lipase-catalysed esterification with alcohols added in work-up).

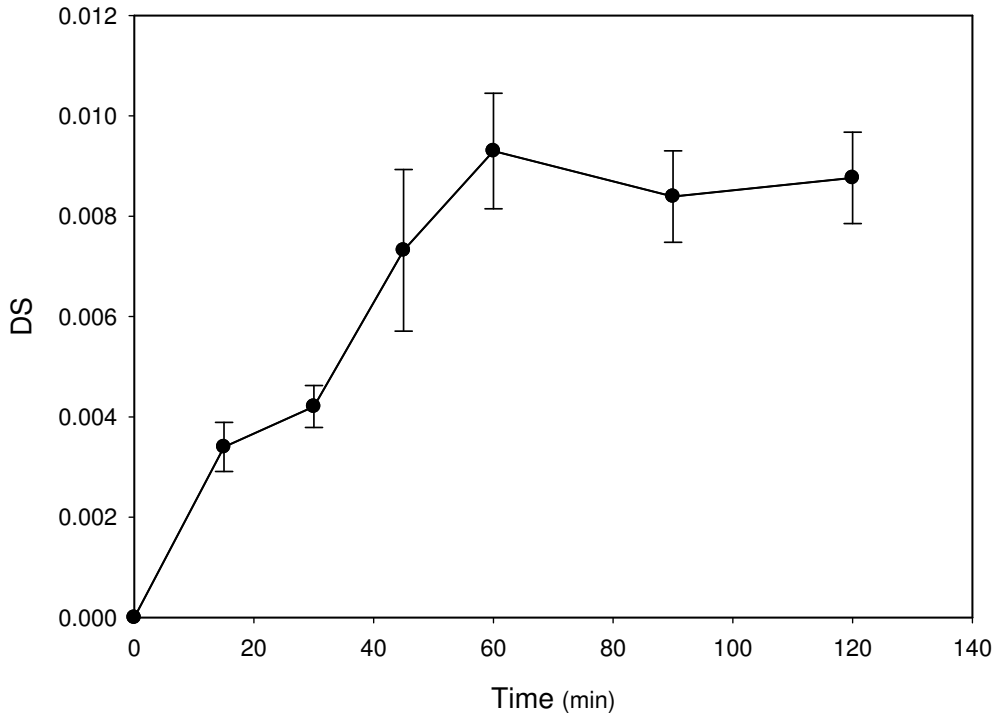

Figure 4.4 Enzymatic synthesis of decanoic acid tapioca starch ester (50^{o}C), progress with time.

4.3.5 Effect of water concentration in gelatinisation mixture

Because water plays an important role in both stages of the process, as a gelatinisation medium and as an enzymatic reaction solvent, the effect of water concentration in the gelatinisation mixture was also investigated. It seems that although the enzymatic reaction as expected is favored by reduced water concentration (or increased starch concentration), a minimum amount of water is necessary for the progression of gelatinisation. As seen in Figure 4.5, initially,

lowering of the water concentration improves the obtained DS after one hour of reaction, however a sudden drop in DS is observed below a certain limit. It can be supposed that this is the minimum amount of water required for all of the starch in the reaction to be gelatinised, a process required for the formation of the aqueous/polysaccharide gel phase. This seems like a reasonable interpretation of the optimum observed in Figure 4.5, which displays this trade-off between increased enzymatic synthetic activity at lower water concentrations and complete disruption of the granular structure at higher water concentrations.

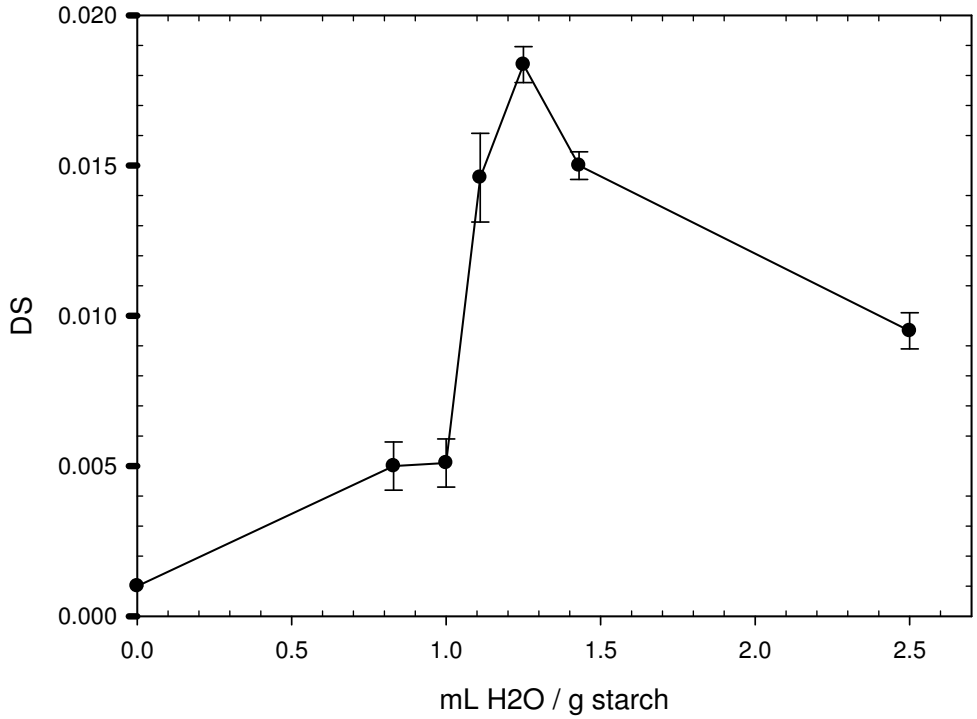

Figure 4.5 Effect of water concentration in the gelatinisation step on the DS, for the subsequent lipolase catalysed esterification of decanoic acid and tapioca starch (60 min, 50°C, 0.5 mL of aqueous lipase solution per g of starch was added after gelatinisation and is therefore present in all systems).

4.3.6 Effect of water in enzymatic reaction

The point made above is further supported by the increased substitution obtained when the enzymatic reaction was run with "pregelatinised starch". For this system tapioca starch was initially gelatinised with phosphate buffer (40% w/v) as normal

and then precipitated directly with acetone. The gelatinised starch was then dried and used as the substrate of the usual enzymatic reaction (same ratio of lipase solution and fatty acid). This reaction was ended and the product retained as normal. The measured substitution was of similar degree (DS=0.013) as that observed for the reactions with the lower water concentrations in the gelatinisation mixture (4.3.4), however the experiment was less reproducible and the error was significantly higher (±0.004). Again the increased substitution can be attributed to the lower water content in the enzymatic reaction (only the enzyme buffer is present), which pushes the equilibrium further towards synthesis of the ester. The drop in precision is potentially a result of the difficulty in reproduction of the starch gelatinisation and drying, as well as the nature of starch in the reaction mixture which resembles more a solid and less a homogeneous gel.

The importance of gelatinisation as an "activating" step to render starch more susceptible to acylation is evident, when this result is compared to the data point for dry starch in Figure 4.5. In this case the enzymatic reaction was performed on dry ungelatinised tapioca starch and the DS obtained was very low (0.001). However, as mentioned, when the same reaction is employed with "pregelatinised" starch, the observed acylation is much higher (0.013 ±0.004).

The same observation was made for the reaction with 40% w/v starch in water. If the starch is not gelatinised prior to enzymatic reaction (so the enzymatic system contains starch dispersed in aqueous buffer, 40% w/v, as well as lipase solution and fatty acid), no acylation could be detected (as opposed to the gelatinised starch reaction where DS of 0.009 was obtained for 40% w/v gelatinisation mixture).

4.3.7 Use of "activated acyl donor"

As mentioned, in many cases the use of "activated" fatty acid derivatives as substrates for hydrolase catalysed ester synthesis, may be favorable. Fatty acid esters are such molecules, with which the initial rate of the biocatalyst is usually increased, since the reaction becomes a transesterification. Vinyl esters in particular may help drive the equilibrium towards synthesis, since the enol produced from the transesterification tautomerises to the volatile acetaldehyde, which moves to the gas phase (Figure 4.6). For this reason the reaction described was attempted with the use

of vinyl ester in place of decanoic acid (same molar concentration). However no difference in the DS was observed after 1 hour of reaction.

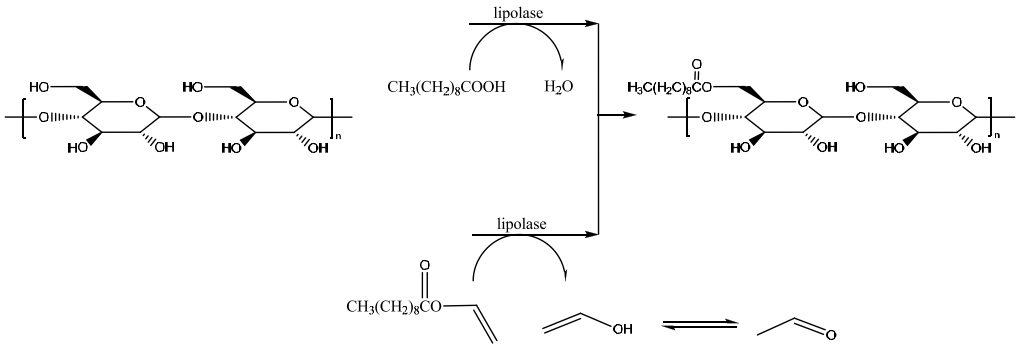

Figure 4.6 Reaction scheme for lipolase catalysed starch decanoylation, with decanoic acid or vinyl decanoate

4.3.8 Other observations

Furthermore we found the presence of surfactant unnecessary for the reaction to proceed, as similar DS were recorded for surfactantless reactions and systems with Triton X-100 used in literature (Rajan & Abraham, 2006; Rajan *et al.*, 2006; Rajan *et al.*, 2008). Presumably the viscous nature of the aqueous phase is enough to ensure adequate dispersion of the fatty acid. It is safe to assume that a system with fatty acid droplets dispersed throughout an aqueous gel is not favoured thermodynamically, thus, given enough time, the two phases will separate. However, it can be perceived, that in a more viscous gel, reassociation of the hydrophobic droplets will be hindered due to the strength of the gel, making the separation process slower.

It should be noted that the product of these reactions could be roughly separated into two fractions, based on their physical appearance. One fraction retained the powdery composition of native starch, while the other fraction was large particles of aggregated starch, possibly containing some other reaction ingredients that could not be removed by washing. This made the dissolution of the whole product in DMSO, prior to sampling for analysis, even more important in order to obtain reproducible and accurate measurements of bulk acylation. This fractionation was more profound for higher amylose contents (see 4.3.2, Figure 4.7). Substitution measured from the powdery fractions were normally two or three times higher than those of the lumpier fractions, however it must be noted that these measurements were not very

reproducible, as we have seen that sampling from solid product leads to low precision.

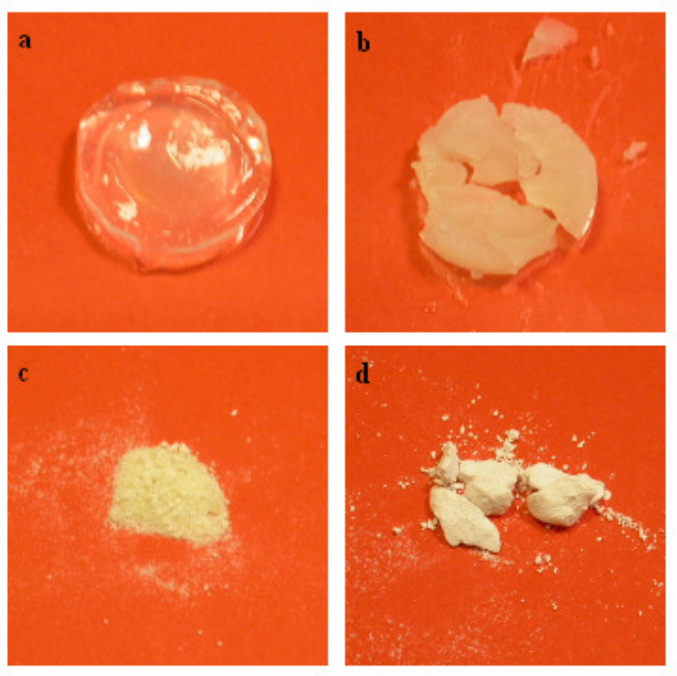

Figure 4.7 Gelatinised tapioca (a) is clear and sol-like while wheat starch (b) is opaque and lumpy. The resulting product is more powdery in the case of tapioca starch (c), while wheat starch produces lumps (d) that can not be easily broken up.

4.4 Conclusions

Lipases are able to catalyse the synthesis of starch esters in the appropriate reaction system. The dispersion of fatty acid throughout an aqueous enzymatic phase, highly concentrated in gelatinised starch coupled with mechanical mixing, was found adequate for the promotion of enzymatic synthesis of starch esters to useful extents (DS ~0.01-0.02). Neither use of surfactant nor fatty acid ester improved the DS of the resulting starch ester. It is noteworthy that starch does not require to be truly dissolved in order to be attacked by the acyl-enzyme intermediate. However ungelatinised starch suspended in the aqueous phase will not react. The reaction products were analysed with the transesterification/GC scheme described previously (chapter 2).

4.5 References

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Chapter 5

Enzymatic acylation with 10-undecynoic acid

5.1 Introduction

As seen in chapter 4, starch esters of decanoic acid can be prepared with the aid of lipase catalysis, from the free fatty acid, in a concentrated aqueous system of gelatinised starch. Starch esters of medium to long chain saturated fatty acids however have been prepared non-enzymatically (Aburto *et al.*, 1999) and have not attracted much attention, mainly because acetylated starch presents more desirable properties for intended uses (Jarowenko, 1986).

Nonetheless it is very interesting to investigate the potential of attaching substrates that the lipase will accept as acyl-donors. The diversity of available fatty acid derivatives potentially makes this reaction system a very useful tool. A very similar substrate to the fatty acid already investigated is 10-undecynoic acid. This is an unsaturated fatty acid, with a chain that is longer by one carbon and that has a triple bond in the ω position.

Ever since Sharpless and co-workers (Kolb *et al.*, 2001) published a landmark paper describing a novel strategy for organic chemistry, employing reliable and selective simple reactions usually leading to carbon-heteroatom bonds, the field has received much interest. The term "click-chemistry" was coined to describe this, while the spearhead reaction of this approach is the Huisgen 1,3-dipolar cycloaddition of alkynes and azides to yield 1,2,3-triazoles.

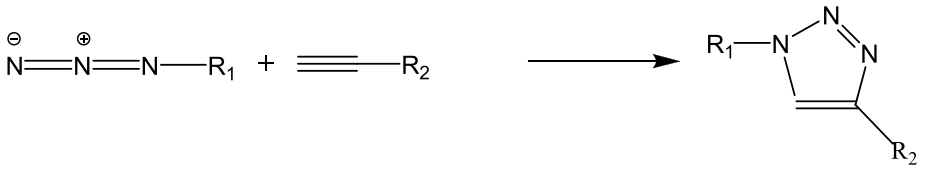

Figure 5.1 Huisgen 1,3-dipolar cycloaddition of a terminal alkynes and an azide to yield a 1,2,3-triazole.

This reaction proceeds readily under diverse and mild reaction conditions and a review of functionalisations of polymers with a wide array of different molecules has recently been published (Binder & Sachsenhofer, 2007), highlighting the potential of this approach. The attachment of an alkyne to a polymeric backbone allows further grafting with molecules that contain an azide group. Thus the alkyne can act as a

linkage for grafting a broad range of other molecules onto the polymer chain. This could be the case if an ester of 10-undecynoic acid with starch is prepared. The esterified alkyne group could potentially act as a grafting site for the attachment of an array of different functionalities to the starch chain.

Here the potential production of starch esterified with 10-undecynoic acid, that may act as a dipolarophile for cycloaddition with azides, is investigated.

5.2 Materials and methods

5.2.1 Materials

10-Undecynoic acid, potato amylose and lipase from *Thermomyces lanuginosus* (lipolase, L0777) were purchased from Sigma (UK). Tapioca, Amioca and Hylon VII were gifts from National Starch (Germany), wheat and maize starches were gifts from BASF (Germany).

5.2.2 Enzyme catalysed synthesis of 10-undecynoyl starch in concentrated aqueous medium

A required amount of sodium phosphate buffer (pH=7.0, 100 mM) is mixed with 2 g of starch (dried overnight in an oven at 60^{o}C), in a 50 mL round-bottom flask. The mixture is incubated in a water bath, for 1 hour at approximately 90^{o}C, while mechanically mixed with a Heidolph overhead stirrer (anchor paddle, 50 rpm), thus gelatinising the starch and then cooled down to 50^{o}C. Next 0.5 mL of lipolase (50 kU, 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 ^{o}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 hour and left overnight at a low temperature (40-50^{o}C) in a convection oven. Alternatively some reactions were ran on smaller scale (100 mg of starch), in 2 mL screw-top Eppendorfs. 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.

DS quantification was performed by methanolysis/GC analysis, as described in chapter 2. Transesterification conditions and GC method are identical. In this case the 10-undecynoic acid methyl ester has retention time of 0.61 min, which means that n-tridecane with a retention time of 0.47 min is used as an internal standard. Fatty acid methyl ester elution time was confirmed with standard produced as described in section 2.2.4. The quantification was based on the theoretical RRF factor, based on the proportionality of detector response and number of "active carbons", which is considered accurate. Possible corrections due to unsaturation were considered unnecessary, as they have been found smaller than other experiment errors (e.g. peak integration, injection) (Ackman & Sipos, 1964; Bannon *et al.*, 1985, 1986).

Errors were calculated as the standard deviation of experiments performed in triplicate.

5.3 Results and discussion

5.3.1 Starch type

The lipolase catalysed esterification of starch with 10-undecynoic acid was tested for a range of different starch types (Figure 5.2). The degree of esterification followed the same observed profile as for decanoic acid, with increasing DS values for higher amylopectin ratios.

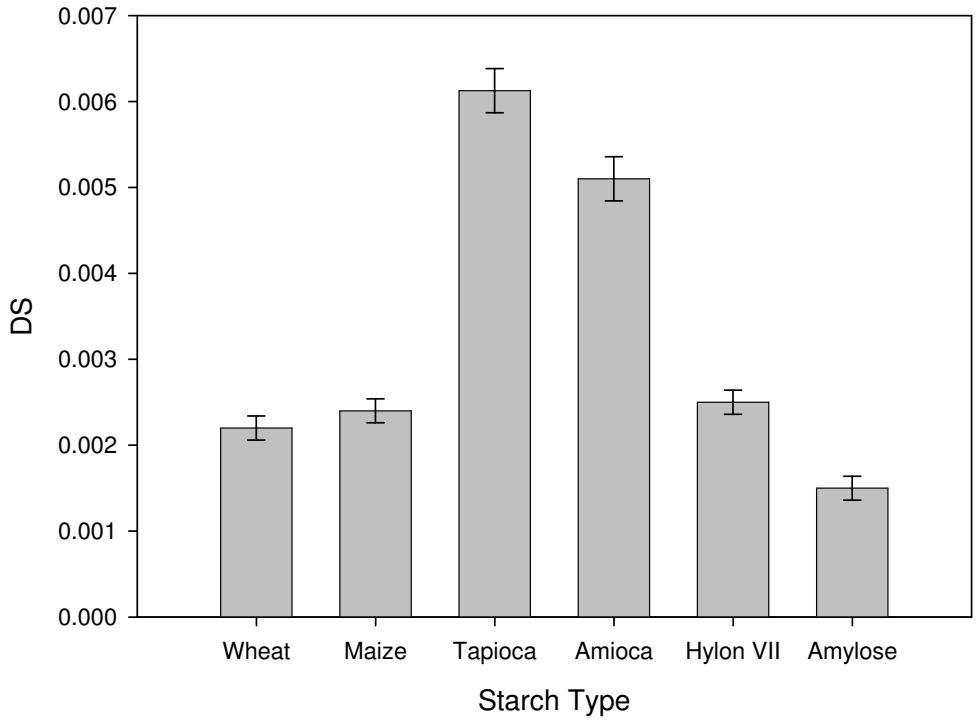

Figure 5.2 DS values for the enzymatic synthesis of starch 10-undecynoate, for different starch types.

5.3.2 Acylation progress

As observed in the case of decanoic acid, the time-course profile (Figure 5.3) seemingly reaches an equilibrium after 1 hour for tapioca starch (shorter time for wheat starch with lower DS).

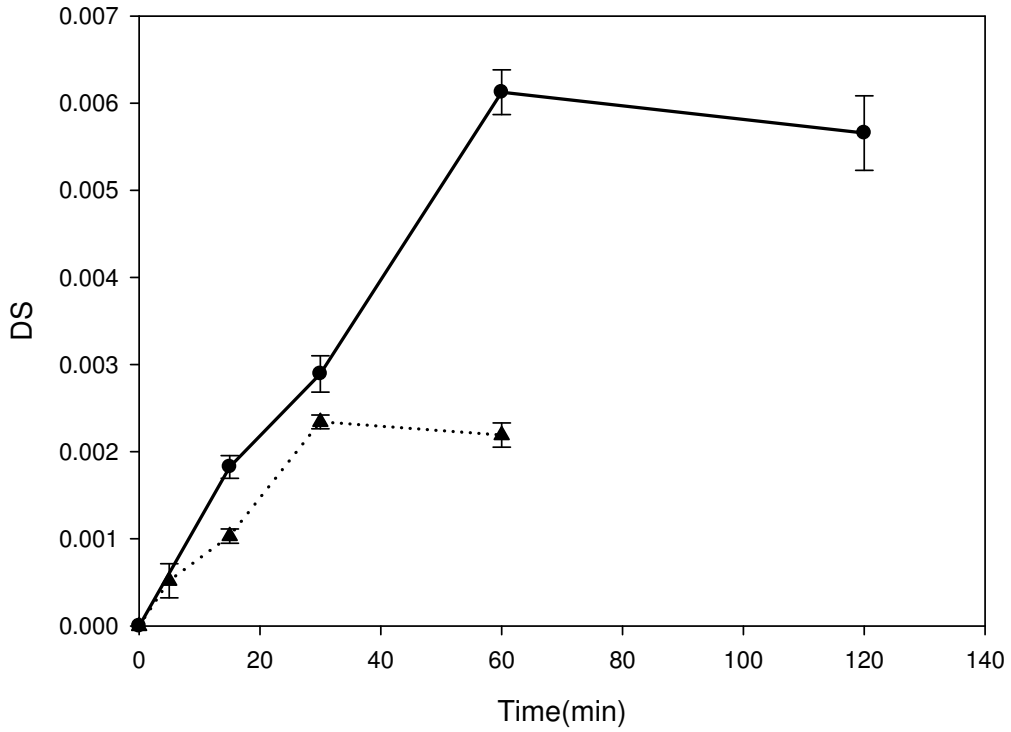

Figure 5.3 Progress of enzymatic synthesis of 10-undecynoic acid esters of tapioca (—) and wheat (•••) starch with time.

5.3.3 Effect of water concentration in gelatinisation mixture

Also the effect of water concentration in the gelatinisation mixture on the DS is observed in this case (Figure 5.4). The DS is altered with small changes of the initial water content, however below a certain water concentration there is a sudden drop in the measured DS. The error in the area of this drop is also relatively high, which also demonstrates how strongly the water concentration affects the DS. It is not always easy to accurately replicate systems with the same water content after gelatinisation, therefore the differences in DS, caused by small differences in water content, give rise to a large standard deviation.

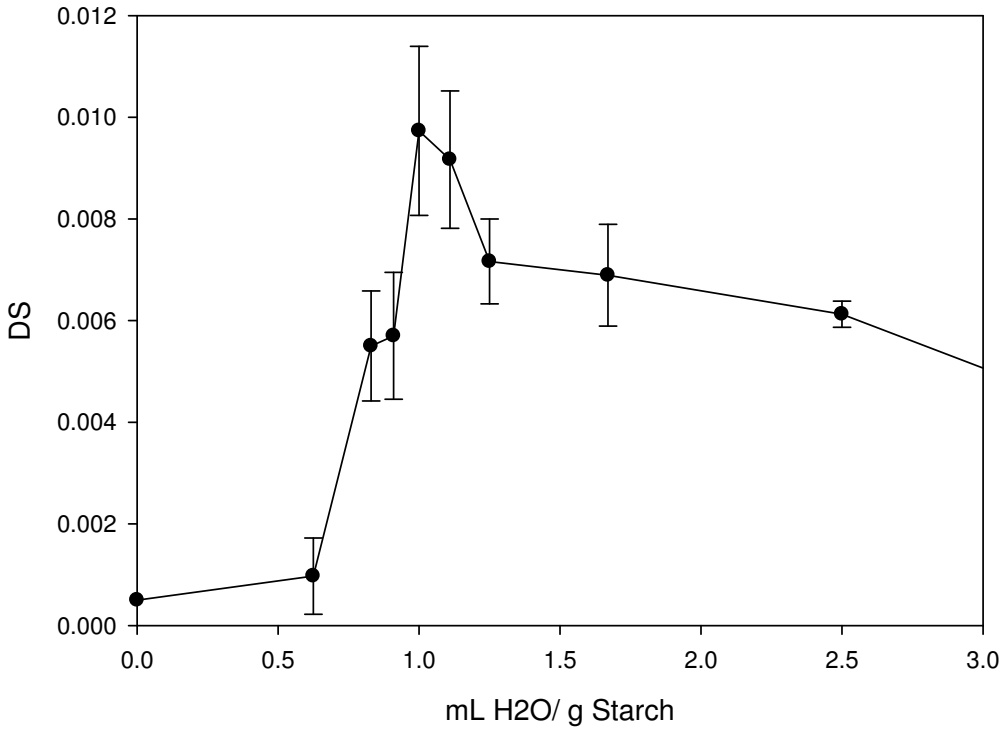

Figure 5.4 Effect of water concentration in the gelatinisation step on the DS, for the subsequent enzymatic synthesis of tapioca starch 10-undecynoate (incubation of 60 min).

5.3.4 Effect of initial concentration of fatty acid

In Figure 5.5 the effect of the initial concentration of fatty acid on the degree of acylation can be seen. It is possible to obtain higher substitutions, after one hour, for higher initial concentrations of fatty acid, but there is an optimum. The decrease in DS for higher concentrations shows an inhibitory effect. This may be direct inhibition of enzyme action by the substrate (concentration of fatty acid in aqueous phase will remain the same, so in this case the inhibition would be related to the action of lipase at the interface) or an effect on the mixing of the two phases present.

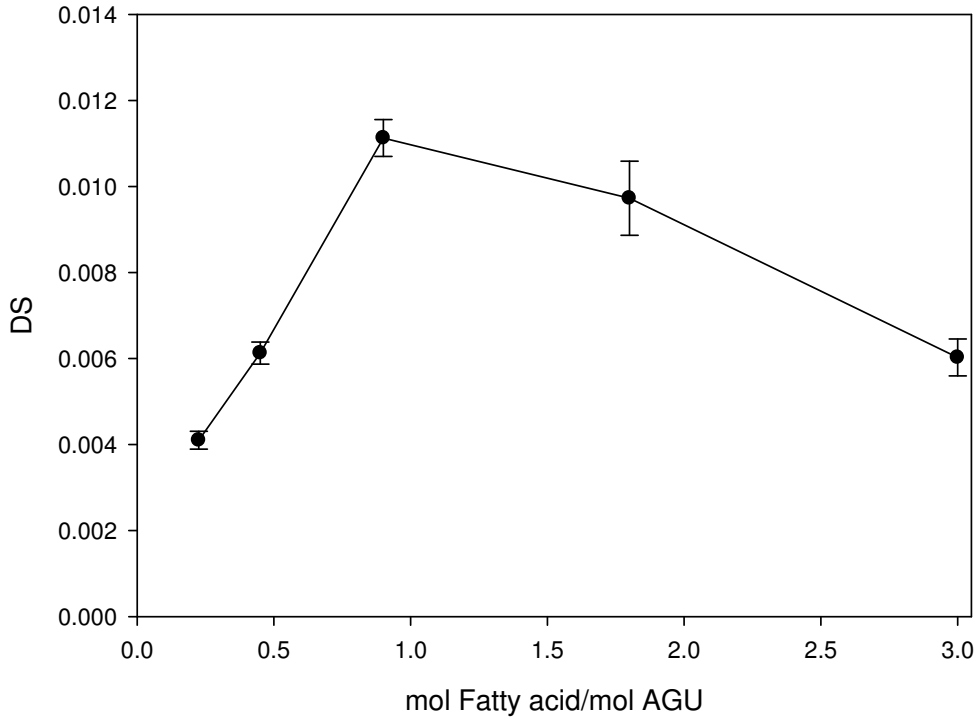

Figure 5.5 Effect of initial 10-undecynoic acid concentration on the lipolase catalysed esterification of tapioca starch, after 1 hour.

5.4 Conclusions

Starch ester of 10-undecynoic acid was prepared by lipolase catalysed acylation in concentrated aqueous gelatinised starch medium. The effect of reaction parameters on the resulting substitution was investigated. Similar to the case of decanoylated starch, tapioca starch resulted in higher substitutions, the reaction reached a plateau after one hour and there was an optimum observed for water concentration in the gelatinisation mixture as well as initial concentration of fatty acid. It was concluded that starch can be acylated to useful extents (DS 0.01) for the potential use of the acyl groups as grafting sites for 1,3-dipolar cycloaddition of molecules with azides.

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Chapter 6

Starch acylation in DMSO

6.1 Introduction

Enzymes, in the vast majority, have evolved to work in aqueous environments, however this does not mean that they may not remain catalytically active in non-aqueous conditions (Zaks & Klibanov, 1984, 1985). Ever since the realisation that certain lipases retain their catalytic activity in anhydrous organic solvents, their use as reaction media to carry out hydrolase catalysed syntheses in, has been very popular.

However in the case of carbohydrate modification, the poor solubility of these molecules in apolar solvents has been one of the main limiting factors of the reactions. In the case of polysaccharides it is obvious that dissolution is going to be an even greater limiting factor. Starch in particular, as mentioned previously, is insoluble in most liquid solvents. The only conventional solvent that is known to dissolve it, albeit in low concentrations, is dimethyl sulfoxide (Leach & Schoch, 1962). The process governing starch dissolution in DMSO seems to be inherently different to the gelatinisation process. The granules do not swell, but rather dissolve by surface peeling or granule fragmentation. DMSO acts as a powerful hydrogen bond acceptor, breaking associative bonds between polysaccharide molecules. The presence of a small quantity of water increases the solution rate in DMSO. This is not understood, but it is thought that the dissolution pathway in the presence of water resembles more the gelatinisation procedure, with swelling of the granule prior to dissolution (Dona *et al.*, 2007).

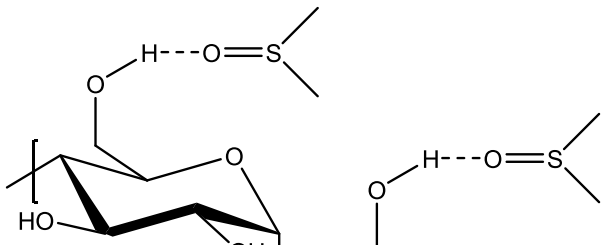

Figure 6.1 The oxygen in DMSO acts as a hydrogen bond acceptor, thus disrupting non-covalent intermolecular bonding of glucans, resulting in dissolution of starch.

So dimethyl sulfoxide is the only reaction medium in which starch could partake as a dissolved substrate. DMSO is an extremely polar and hygroscopic organic solvent. This could mean that a possible DMSO anhydrous reaction system will require removal of water. However, like many water-miscible solvents, it poses an even greater problem for enzymatic catalysis, as it is well documented that addition of DMSO to enzyme solutions causes loss of catalytic activity. Furthermore, for close to 100% organic solvent, unlike most systems where enzymes are suspended and active, in DMSO they are usually dissolved and denatured (Jackson & Mantsch, 1991; Simon *et al.*, 1998). For this reason enzymes used in DMSO are normally somehow protected (Ge et al., 2009), in order to inhibit the stripping of essential water molecules from the protein surface that is thought to take place. It is also obvious from literature that DMSO actually breaks intramolecular associations in the enzyme, disrupting the secondary structure and in high concentrations completely unfolds the protein (Jackson & Mantsch, 1991). There is in any case much interest on enzymatic synthesis in DMSO, mainly because of its ability to dissolve polysaccharide material. However most reports have to be approached with caution, due to the discrepancies we have found in analytical methods. Yang et al. reported synthetic activity of Amano lipase A12 in DMSO, for the acetylation of cellulose (Yang & Wang, 2003). Qiao *et al.* (Qiao *et al.*, 2006), used lipases to improve esterification of alkenyl ketene dimers to starch. In both cases, the degree of acylation was low.

We investigated the possibility of acylation of starch with fatty acid in DMSO based systems, catalysed by enzymes.

6.2 Materials and methods

6.2.1 Reagents

Candida antarctica lipase B, immobilised on acrylic resin (Novozyme 435, L4777), Amano lipase A (from *Aspergillus niger*, 534781) and anhydrous dimethyl sulfoxide were purchased from Sigma (UK). Acetylated starch (Eurylon G) was a gift from Roquette (UK).

6.2.2 Acylation in DMSO

Native starch (dried overnight at 60°C) was mixed with DMSO (anhydrous grade) in order to produce a solution or dispersion, depending on the concentration. To this mixture the enzyme (solution for lipolase, dry powder for Amano A, particles for immobilised CALB) was added along with the acyl donor (decanoic acid or vinyl decanoate). The reactions, scaled to fit 2 mL microcentrifuge tubes, were incubated at 50°C in an air incubator and mixed by inversion. After completion, the starch was precipitated from the mixture by tenfold dilution with acetone. The resulting precipitate was further washed with the same volume of acetone twice, before being dried in a desiccator under vacuum (approx. one hour) and overnight in a convection oven (40-50°C). Non-enzymatic controls were run, where no enzyme was present or buffer was added instead of enzyme solution.

6.2.3 GC analysis of vinyl ester content

The amount of vinyl ester present in the reaction solution was monitored on a Perkin-Elmer Autosystem XL GC-FID with a CP Simdist capillary column (oven at 100°C, injector at 110°C, detector at 130°C). The reaction solution was injected directly into the GC and the vinyl ester peak was observed, after the DMSO front.

6.3 Results and discussion

6.3.1 Acylation in DMSO

Several variations of a possible system for enzymatic DMSO acylation were tested. Anhydrous reactions were carried out with Novozyme 435 and Amano A, while lipolase was used in solution. No starch ester formation could be observed after 24 hours when decanoic acid was used as the acyl donor. The addition of a DMSO miscible organic solvent, such a *t*-butanol (33% v/v), did not help in retention of enzymatic activity. It is also worth noting here that no acylation could be detected in pure *t*-butanol either. This is not surprising since both starch and enzyme are in suspension in such a system. The use of commercial acetylated starch as starting material (presumably is more soluble in polar organic solvents) also did not result in

acylation. This may be due to low DS of the commercial derivative (0.01-0.1) which does not impart the starch with enough hydrophobicity, to be soluble in *t*-butanol.

The use of vinyl decanoate as the acyl donor resulted in seemingly paradoxical results. Ester formation was clearly observed for the enzyme catalysed reactions, however the DS obtained for the non-enzymatic controls had an ever higher value (Table 6.1).

Starch type	Enzyme	DS
Wheat	Lipolase (0.5 μL per starch mg, 50 Units)	0.036
Wheat	Immobilised CALB (0.8 mg per starch mg, 8 Units)	0.005
Wheat	Amano A (0.17 mg per starch mg, 2 Units)	0.085
Wheat	None	0.198

Table 6.1 Acylation of starch with vinyl decanoate (2 mol per mol anhydroglucose) in DMSO (6%w/v)

Alteration of the washing procedure was employed to inhibit any potential carry over of residual vinyl ester to the methanol transesterification reaction (for FAME formation prior to GC analysis). Introduction of extra washing steps before and after precipitation, with *n*-heptane or diethyl ether, had no effect on the observed DS values.

The vinyl ester content in the reaction solution was monitored, by GC analysis with direct injection.

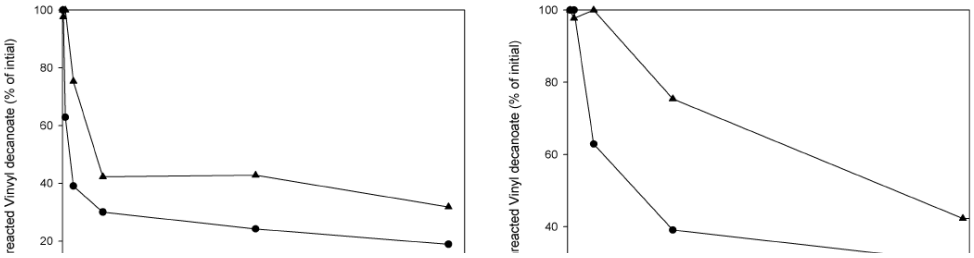

Figure 6.2 Vinyl decanoate concentration in DMSO reaction solution for enzymatic (▲) and control (●) systems. The concentration is shown for different timescales.

From the results in Figure 6.2, it is obvious that vinyl ester is indeed being consumed through the course of the reaction, as the concentration is obviously dropping. This probably rules out simple carry over of vinyl ester to the alkaline methanolysis reaction. One explanation for the lower DS values obtained for enzymatic systems could be that the enzyme hydrolyses some vinyl ester, forming decanoic acid which does not readily acylate the starch. The enzyme could also be catalysing the hydrolysis of the starch ester that has formed non-enzymatically. However in both these cases, one would expect higher or equal rate of vinyl ester consumption for the enzymatic systems, even though it is not going towards starch ester synthesis. This is not the case. The enzymatic system shows a slower vinyl ester consumption, compared to the non-enzymatic control. It seems the presence of the enzyme alone is enough to inhibit the transesterification.

This reaction system is obviously more complicated that it first appears, however the lack of clear enzymatic acylation of the polysaccharide deems any further investigation out with the scope of the current thesis.

6.4 Conclusions

Despite several reports in literature of successful enzymatic esterification of starch in DMSO, we could not detect product in the case of lipase catalysed starch decanoylation, as decanoic acid failed to work as an acyl donor in pure DMSO.

When the vinyl ester was used, starch ester was detected however the DS values obtained for the enzymatic systems were lower than those measured in the non-enzymatic controls.

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Chapter 7

Enzymatic acylation of starch in ionic liquids

7.1 Introduction

Ionic liquids are salts, usually composed of an inorganic anion and an organic cation, that remain liquid in conditions close to room temperature. Ionic liquids can be formed between a large number of different interchangeable ions, which leads to liquids with diverse properties. For this reason they are often termed as designer solvents. Another term often used for these salts is "green solvents" (Earle & Seddon, 1998), mainly due to their low vapour pressure, which minimises air pollution, an important setback for many organic solvents. However, the similarities between many ionic liquids begin and end with their liquid salt nature, as their properties could be as diverse as those found between members of other broad compound classifications, such as organic solvents. It must therefore be noted that the lack of information on toxicity is sometimes overlooked, which may mean that a specific ionic liquid could be a very potent water or land pollutant, despite its minimal vapour pressure.

The first popular ionic liquids have had in common low vapour pressure and the ability to dissolve a broad range of substrates. These properties attracted the attention of synthetic chemists, since they are in many cases highly desirable in a solvent (Handy & Zhang, 2001). Enzymatic catalysis often takes place in systems where substrates and products display important differences in hydrophobicity (Zaks & Klibanov, 1984). One such case, as mentioned, is the synthesis of sugar ester derivatives, with the reverse action of hydrolases. The solubility of sugars in polar solvents is often countered by the hydrophobicity of the other substrate. Longer chain fatty acids for example, are sparingly soluble in water. As seen previously, organic solvents capable of dissolving both (e.g. pyridine, *tert*-alcohols etc), generally do so at low concentrations and often lead to enzyme denaturation. For this reason multi-phase systems are used, where a large array of imaginative tricks are employed to bring the reactants in contact. However here is a type of solvent that will dissolve all substrates in adequate quantities, to get decent yields. This has prompted enzyme chemists to research the activity of enzymes in such solvents, with diverse results (Yang & Pan, 2005). In many cases lipases retain their activity, especially in immobilised form, however this is usually in less polar ionic liquids with reduced

sugar solubility. At least for the more widely available ionic liquids, there is a trend between higher carbohydrate solubility and enzyme inactivation. An attempt to predict enzyme behaviour in RTILs, was based on the Hoffmeister series as a measure of ion effect on protein stability (Zhao, 2005). This approach led to successful predictions for ionic liquid mixtures with water, but could not predict the enzyme behaviour in pure ionic liquids. One could argue that this is expected, since ionic liquids in water may simply act as ionic solutions. Of course the lack of a reliable general model for enzyme behaviour prediction, did not stop reports of successful enzymatic syntheses in ionic liquid containing systems, from appearing in the literature. Erbeldinger *et al.* (Erbeldinger *et al.*, 2000) reported the first biocatalytic reaction in an ionic liquid, the synthesis of Z-aspartame in 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]) catalysed by thermolysin. A number of publications followed suit (Kim *et al.*, 2003; Kragl *et al.*, 2002; Park & Kazlauskas, 2001; van Rantwijk *et al.*, 2003) and it was not long before the first report of a sugar ester synthesis in an ionic liquid catalysed by immobilised CALB, by Ganske and Bornscheuer (Ganske & Bornscheuer, 2005a). After optimising, the same group reported 60% conversion of fatty acid vinyl ester and glucose to the sugar ester, by immobilised CALB, with addition of t-butanol as a second phase (Ganske & Bornscheuer, 2005b).

This prompted a fair amount of research into this possibility, but again there were a number of problems to overcome. The relatively low solubility of sugars in the ionic liquids that enzymes retained their synthetic activity in meant that though conversions might be high, the processes may not be practical (Liu *et al.*, 2005). Because of the ability of RTILs to dissolve such a wide range of materials, the isolation of the product can be extremely difficult. Researchers have tried to overcome these hurdles by inventing and investigating the potential of different types of ionic liquids, by trying to run reactions in supersaturated solutions (Chen *et al.*, 2006; Lee *et al.*, 2008a), or by using mixtures of ionic liquids (Lee *et al.*, 2008b). In this way sugar esters were prepared in relatively high conversions and even konjac glucomannan was reportedly acylated with short chain fatty acid vinyl esters (Chen *et al.*, 2006). Extremely hydrophilic RTILs, such as chloride anion RTILs, that have great carbohydrate dissolving potential seem to inactivate biocatalysts. This may be

similar to the effect of polar organic solvents such as DMSO, that strip essential water off the surface of enzymes (Moniruzzaman *et al.*, 2007), or they may well dissolve the enzyme, unfolding its active conformation (Jackson & Mantsch, 1991). Again there must be a trade-off between enzymatic activity and carbohydrate solubility and new RTILs with acetate or phthalate anions may be able to combine the two properties at satisfactory levels. Also dicyanamide anion RTILs show promise, as they reportedly will dissolve polysaccharides while at the same time can act as solvent for the Novozyme 435 catalysed synthesis of sucrose dodecanoate (Liu *et al.*, 2005).

In this chapter the possibility of applying systems with RTILs that reportedly dissolve polysaccharides to a certain extent , for the enzymatic acylation of starch is investigated.

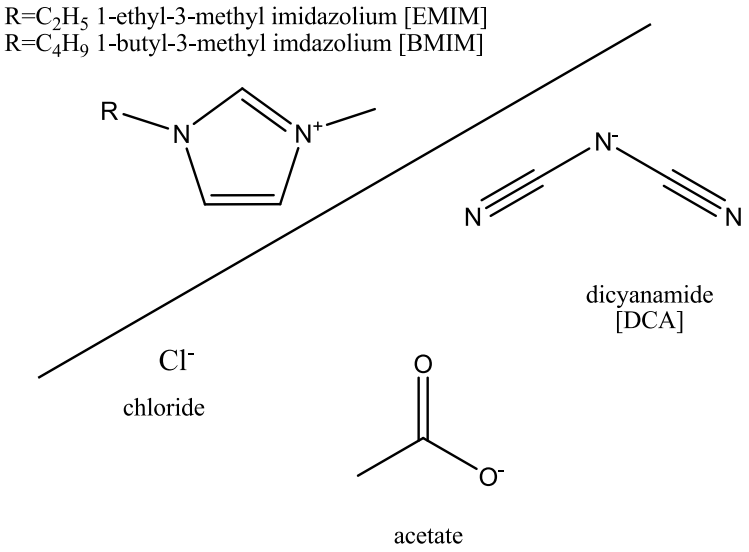

Figure 7.1 Ions present in ionic liquids used for the present study. Chloride anion ionic liquids are good solvents for polysaccharides. Acetate and dicyanamide RTILs show particularly low viscosity.

7.2 Materials and methods

7.2.1 Materials

Wheat and maize starch were gifts from BASF (Germany), tapioca starch, Amioca and Hylon VII were gifts from National Starch (Germany). Lipase from *Thermomyces lanuginosus* (lipolase, L0777), immobilised *Candida antarctica* lipase B on acrylic resin (Novozyme 435, L4777), Amano lipase A (from *Aspergillus niger*, 534781), Amano lipase PS (from *Burkholderia cepacia*, 534641) were obtained from Sigma (UK). 1-Butyl-3-methyl imidazolium chloride ([BMIM][Cl]), 1-Ethyl-3-methylimidazolium chloride ([EMIM][Cl]), 1-Ethyl-3-methylimidazolium acetate ([EMIM][CH_3COO]) and 1-Ethyl-3-methylimidazolium dicyanamide ([EMIM][DCA]) were gifts from BASF (Basionics, Germany) and were used without further treatment.

7.2.2 Enzyme catalysed starch acylation in RTILs

A mixture of dry starch and ionic liquid was prepared in a suitable vessel. Most reactions of such type were performed in 2 mL screw-top microcentrifuge tubes, with 50 mg of starch. To this an appropriate amount of lyophilised, dissolved or immobilised enzyme was added. Fatty acid or the according vinyl ester were preheated to the reaction temperature and added to the reaction mixture. Finally the appropriate enzyme was added. The system was then mixed thoroughly, by vortex. The reactions were incubated in an air-incubator, while mixed by inversion. After the reaction completion, the starch was precipitated by tenfold dilution with an organic solvent (ethanol or acetone) and then washed twice with the same volume of the same solvent. Finally the product was dried under vacuum in a desiccator. The resulting starch esters were analysed as described in chapter 2, by transesterification/GC analysis.

7.3 Results and discussion

7.3.1 $[Cl]^-$ anion RTILs

Two different ionic liquids with a Cl^- anion were tested as suitable solvents for enzymatic acylation of starch, 3-ethyl-1-methylimidazolium chloride and 3-butyl-1-methylimidazolium chloride ([EMIM][Cl], [BMIM][Cl]). The chloride anion ionic liquids are known to be good solvents for polysaccharides and have been used as media for non-enzymatic modification of cellulose and starch (Biswas *et al.*, 2006; Liu *et al.*, 2006; Remsing *et al.*, 2006; Stevenson *et al.*, 2007).

Reactions were attempted in 10% w/w starch solutions (typically 0.5 mL) at 60°C (the maximum temperature for the air incubator), in order to lower the RTILs' viscosity as much as possible. Both decanoic acid and its vinyl ester were tried as substrate (2 mol per mol anhydroglucose), while *Candida antarctica* lipase B immobilised on acrylic resin (Novozyme 435, 5 Units -sigma assay- per mg starch) was used as catalyst. Our observations confirmed the absence of synthetic activity in chloride anion ionic liquids, as no ester formation could be detected in either the reaction or control systems for [EMIM][Cl] and [BMIM][Cl].

7.3.2 $[CH_3COO]^-$ anion RTIL

Acetate anion ionic liquids are relatively new and have not been thoroughly investigated, but have been known to be "enzyme-friendly"(Zhao *et al.*, 2006), however not much is known about their polysaccharide dissolution capabilities.

Starch type	Ionic Liquid	Enzyme	DS
Wheat	[EMIM]Ac	Immobilised CALB (5 Units per mg starch)	0.151 ± 0.022
Wheat	[EMIM]Ac	None	0.064 ± 0.009
Hylon VII	[EMIM]Ac	Immobilised CALB (5 Units per mg starch)	0.359 ± 0.025
Hylon VII	[EMIM]Ac	None	0.120 ± 0.009

Table 7.1 Acylation of starch in 0.5 mL of pure [Emim][CH_3COO], 10% w/w. with vinyl decanoate (2 mol per mol anhdroglucose), at 60°C for 24 hours, mixed by inversion (units refer to sigma assay).

No product could be identified for the reactions with the free fatty acid substrate. On the other hand we observed that the acetate anion systems where the vinyl ester is used as the acyl donor, resulted in starch esters for both the enzymatic and control systems, with relatively higher DS values for the enzyme catalysed reactions. It is also noteworthy that the DS for the high amylose starch type was much higher than the value recorded for the native starch type.

It is not clear that the polysaccharide is dissolved in the ionic liquid during the reaction. Optically, the mixture seems clear and of the same colour with the pure ionic liquid, but centrifugation will separate out a large amount of the starch, as a distinguishable solid phase, of white powder, forms at the bottom of the tube. This suggests that the polysaccharide is not truly dissolved, at least not all of it, but rather very well dispersed.

7.3.3 $[DCA]^-$ anion RTIL

Dicyanamide ionic liquids have been known to dissolve sugars in large quantities (Liu *et al.*, 2005) although again most enzymes seem to lose catalytic activity in them. Mixing starch and fatty acid with 1-ethyl-3-methyl imidazolium dicyanamide (10%w/w), and incubating for several hours at 60^{o}C results in ester formation, but with very low DS values. Adding lyophilised Amano A lipase (1 Unit –Amano assay- per mg starch) to this mixture however led to the increase of the DS, as seen in Figure 7.2.

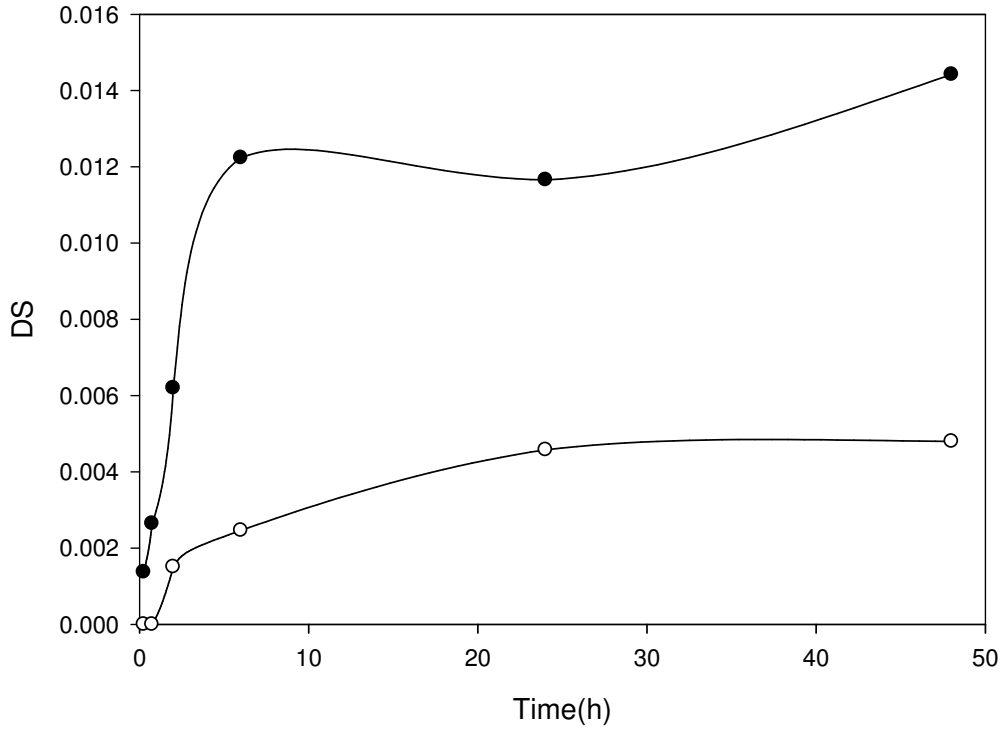

Figure 7.2 Enzymatic esterification of decanoic acid and wheat starch with Amano A lipase (●) and the chemical reaction background (○), with time, in 1-ethyl-3-methylimidazolium dicyanamide (10 %w/w, 0.5 mol decanoic acid per mol anhydroglucose, incubated at 60°C, mixed by inversion).

Figure 7.2 shows the progress of the enzymatic reaction with time, as well as the observed chemical background. After 48 hr the DS for the non-enzymatic reaction in [EMIM][DCA] is similar to the value obtained in enzymatic aqueous systems (after 1hr of reaction, 40% w/v gelatinisation mixture, see Chapter 4), but it is four times greater for the enzymatic reaction in [EMIM][DCA]. It is noteworthy that addition of CALB immobilised on acrylic resin (5 Units per mg starch), does not improve the substitution compared to the chemical background. Finally it was also possible to produce ester in the case of 10-undecynoic acid. With tapioca starch and for the same conditions, a DS of 0.007 was obtained.

Unfortunately there seems to also be a by-product generated by the mixture of the fatty acid with the ionic liquid. This is evident when the reaction mixture is directly inserted into the transesterification (sodium methoxide) solution, with omission of

the acetone precipitation step. When this is done, a large methyl decanoate peak can be detected as the product of the transesterification work-up, which would attribute a DS of approximately 0.05, both in the enzymatic as well as the control mixtures.

This means that either starch ester is synthesised at a higher DS but an amount of the highly substituted starch remains dissolved during the acetone precipitation, or there is something else that results in the synthesis of methyl decanoate during the transesterification analysis. A control where no starch or enzyme is present was incubated and injected into the sodium methoxide, methanolic solution. This indeed resulted in a methyl ester peak, which meant that there is no residual starch ester left over after precipitation, but rather something else is causing the appearance of the methyl ester peak. This could either be some sort of catalysis by the ionic liquid of direct esterification of residual fatty acid onto methanol in the transesterification mixture, or the actual generation of some product during incubation that would then produce the methyl ester after transesterification. A time course of the previous control revealed that for [EMIM]DCA/decanoic acid mixtures that are not incubated there is no generation of methyl ester and the formation of methyl ester actually grows with the time the mixture is left to incubate, prior to transesterification. This makes it obvious that something is actually generated as a by-product of the co-incubation of the ionic liquid and the fatty acid, which can then go on to form the methyl ester, under the alkaline methanolysis conditions. If it were merely a case of the direct fatty acid/methanol esterification being somehow catalysed by the ionic liquid, there would be no difference in methyl ester formation between different incubation times, or at least there could be some methyl ester detected when the mixture is not incubated. This may mean that the DS measured after acetone precipitation is the true DS, as whatever by product is generated, will probably remain in the liquid phase which is discarded. Implications caused by the presence of this by-product makes proving the formation of the ester an arduous task, especially in this case where there is a chemical background detected.

7.4 Conclusions

Ionic liquids do indeed present novel possibilities as solvents for polysaccharides, however in some cases the actual dissolution is questionable. It may be that what is being reported is the potential of dispersion of the polysaccharidic material, which is also observed in very polar organic solvents. Strongly hydrophilic RTILs with starch dissolving capability, such as $[Cl]^-$ anion RTILs, provide a hostile environment for enzymes and lipases do not present activity even when immobilised. Acylation was detected in pure [EMIM][CH_3COO] when vinyl ester was used as substrate, while increased substitution was observed when immobilised CALB was present. Finally, acylation was observed in [EMIM][DCA] when free fatty acid was used as substrate. Addition of suspended lyophilised Amano A lipase leads to four-fold increase in substitution, while immobilised CALB did not improve substitution. However by-product formation between the fatty acid and ionic liquid complicate this system.

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Chapter 8

Further analysis of starch esters

8.1 Introduction

8.1.1 Enzymatic hydrolysis

Enzymatic hydrolysis of starch is a crucial biochemical process in living organisms, since it is the main way in which the stored energy in starch can be turned into a form that can undergo glycolysis, to produce pyruvate and ATP for the Krebs cycle. It is also one of the oldest and most used biotechnological processes. Diastase found in malt liquid, was the first isolated enzyme and is the source of the suffix –ase, used for most enzymes. Though in early texts reported to be a single enzyme it most likely consisted of a range of starch hydrolytic enzymes. For many years researchers believed that diastase may be used by organisms in starch synthesis as well (Onslow, 1920).

Nowadays it is known that there are many different types of starch hydrolysing enzymes (Bertoldo & Antranikian, 2002; Robyt, 1984). Amylolytic enzymes can be broadly categorised into endo- and exo-acting, based on whether they attack the polysaccharide chain in the interior or the end respectively. The most common endo-hydrolases are α-amylases which randomly hydrolyse α-1,4 linkages within the starch chain, resulting in a mixture of branched and linear oligosaccharides and α-limit dextrins. β-Amylases and glucoamylases are exo-hydrolases that attack the non-reducing end of starch chains giving oligo- or monosaccharides. α-Glucosidases further hydrolyse oligosaccharidic linkages to give α-anomeric glucose. A host of debranching enzymes attack the α-1,6 linkages in starch, pullulanases being the most common. They are further divided based on their hydrolysis products and their ability to act upon α-1,4 linkages as well. Glycosyltransferases are a family of enzymes with the ability to break linkages in polysaccharide chains, thus detaching a portion in order to transfer said portion to another glycosyl acceptor. Cyclodextrins are made by glycosyltransferases that attach the two ends of the glucose oligomer obtained, rather than attaching to an altogether different glycosyl acceptor.

Amylases are therefore used to hydrolyse starch in biotechnological applications, but they are also produced by microorganisms in order to degrade a polysaccharidic structure. It is therefore useful to produce modified starches with the ability to resist

amylase digestion. The action of these enzymes is known to be inhibited by the presence of bulky substitutents on the hydroxyl groups of the glucan (Whelan, 1964).

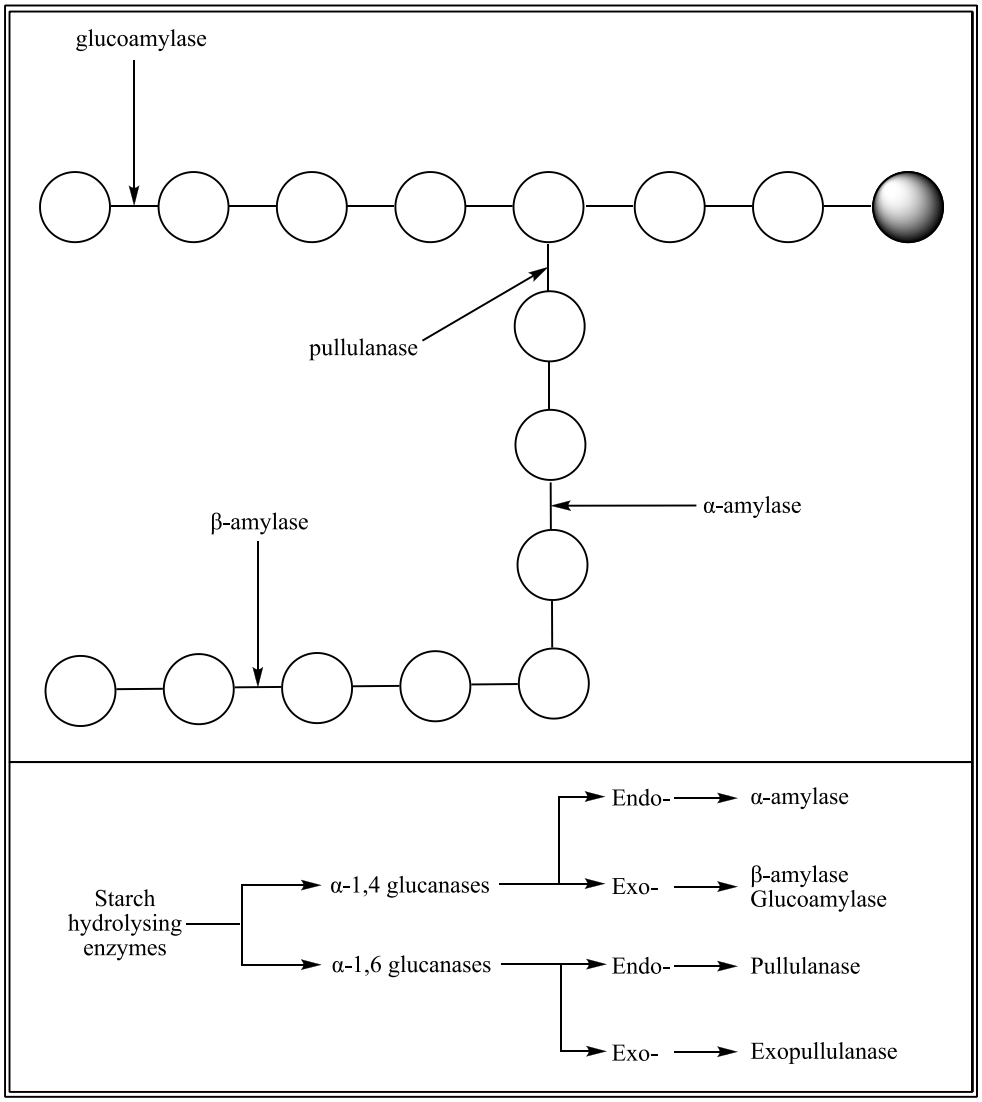

Figure 8.1 General classification of starch degrading enzymes and schematic presentation of their action (shadowed unit represents reducing end).

Esterification therefore is a useful way of creating a steric hindrance to the enzyme's hydrolytic action (Aburto *et al.*, 1999).

Here the inhibition of amylase digestion on the starch esters produced by lipase catalysed esterification, is tested. Furthermore investigation of the enzyme hydrolysis

patterns obtained for different amylases, could elucidate the structure of the acylated starch.

8.1.2 Photograph microscopy

As described earlier, starch is found in plants in a granular structure that ranges in size from a few, to a few hundred μm and occurs in a variety of shapes, depending on the source. Microscopy therefore can be a very useful technique when working with starch, for identifying the source of particular starch samples, as well as observing the phenomena that take place at a microscopic scale such as swelling and gelatinisation. Under ordinary light starch granules appear colourless and clear, with a mean refractive index of 1.5 (French, 1984). It is possible to observe the concentric growth patterns, with regions of alternating high and low refractive index.

Because of the semi-crystalline nature of starch granules, observation under polarised light may be a very effective tool. Starch crystallites exhibit birefringence, typically in the form of a maltese cross. The intensity of observed birefringence depends on the thickness of the granule and degree of crystallinity and orientation of the crystallites

Though the loss of birefringence that characterises gelatinisation and loss of granular structure, is best observed under polarised light, much information can be obtained from an ordinary light microscope. The granule size, source and gelatinisation temperature can all be measured with the aid of a simple light microscope.

Here we present with the aid of photograph microscopy, some observations on the nature of the native starch and the enzymatically produced esters, used in experiments described.

8.2 Materials and methods

8.2.1 Reagents and materials

Sodium phosphate monobasic, sodium chloride, 3,5-dinitrosalicylic acid, sodium potassium tartrate, maltose monohydrate and α-amylase from *Aspergillus oryzae* (10065) were all purchased from Sigma(UK). Disposable 3mL polystyrene cuvettes

were purchased from Fisher Scientific (UK). The spectrophotometer is a Beckman-Coulter DU800 and the analysis was done with DU800 application software.

8.2.2 DNS Reagent

0.438 g 3,5-dinitrosalicylic acid (DNS) is dissolved in 20 mL deionised water (96 mM), with the aid of slight heating and stirring. 12 g of sodium potassium tartrate is dissolved in 8 mL of NaOH 2.0 M, again with aid of slight heating and stirring. Care is taken in both cases not to reach a boiling temperature. The sodium potassium tartrate solution is then added slowly and with stirring to the 3,5,-dinitrosalicylic acid solution and the mixture is diluted to 40 mL. The resulting DNS reagent is stored in an amber bottle, at room temperature and is stable for up to 6 months (Bernfeld, 1955).

8.2.3 α-Amylase assay

This method was based on the amylase assay proposed by Sigma, taken from Bernfeld (1955). The presence of carbohydrate reducing ends is detected by the spectrophotometric measurement of DNS reduction to 3-amino-5-nitrosalicylic acid, which displays an absorbance maximum at 540 nm (Figure 8.2).

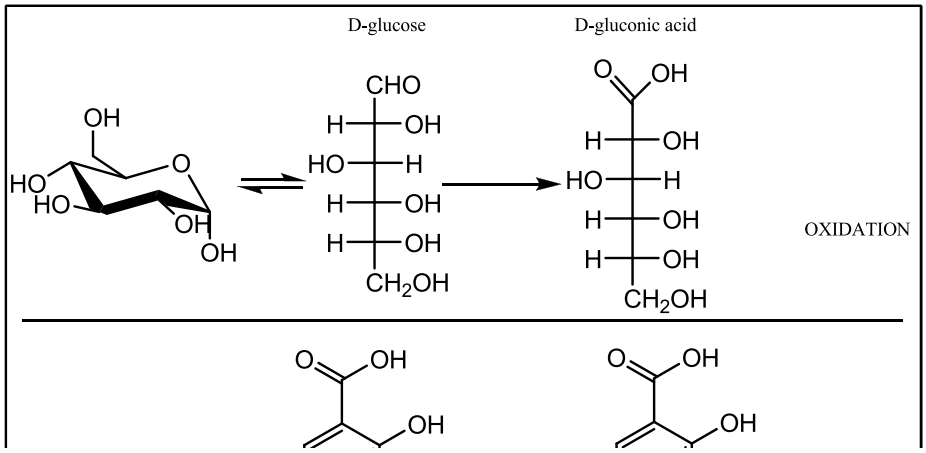

Figure 8.2 The aldehyde of the reducing carbohydrate is oxidised to the carboxylic acid, while DNS is reduced to 3-amino-5-nitrosalicylic acid.

Equal volumes of starch solution (or aqueous suspended particles, 0.03 mmol mL^{-1}) to be analysed (in appropriate buffer) and amylase solution (in 20 mM sodium phosphate, pH=6.9, 6.7 mM sodium chloride) are mixed and incubated at 30°C for the required amount of time. To an aliquot of this solution, half the volume of DNS reagent is added and the mixture is heated at 100°C for 15 min. It is then cooled in ice to room temperature. The sample is then diluted to 4 times its original volume with deionised water and the absorbance is recorded on the spectrophotometer at a wavelength of 540 nm. The volume ratios should be 1:1:1:3 of starch solution : amylase solution : DNS reagent : deionised water. A control is run where deionised water is added to the starch instead of amylase, or where amylase solution is added after the DNS reagent.

The spectrophotometer is a Beckman-Coulter DU800 and the analysis was done with DU800 application software.

8.2.4 Standard Curve

A 0.2% w/v standard stock solution of maltose in deionised water is made and from this five standards are prepared by diluting, in the range of 0.02-0.1% w/v. To each sample, half the volume of DNS reagent is added and the mixture is heated at 100°C for 15 min and cooled to room temperature in ice. The samples are then diluted to four times their volume with deionised water and the resulting solutions' absorbance is measured on the spectrophotometer at 540 nm. The volume ratios should be 2:1:3 of standard : DNS reagent : deionised water.

8.2.5 Photograph Microscopy

Gelatinised tapioca starch was prepared by heating (90°C, 1 hour) of native starch in water and subsequent precipitation with acetone. Starch esters were prepared as described in sections 5.2 and 7.2. In all cases the starch was in a dry particle form prior to analysis.

Starches were observed dispersed in water, on an Olympus Vanox light microscope. The magnification was x400, with the aid of an M40 magnifier (x40) and a Nikon Microcam Relay lens (Olympus, x10). Photographs were taken with a Nikon E4500 camera, attached to the microscope.

8.3 Results and discussion

8.3.1 α-Amylase digestion of starch ester prepared by lipolase catalysis in concentrated aqueous gelatinised starch medium (chapter 4)

As with most enzyme assays, there is a variety of ways in which the enzyme activity can be measured and expressed. Amylases are no different. The physical properties of the polysaccharide substrate as well as the way that it will be treated prior to assay, can be varied. In many cases soluble starch substrate is gelatinised in dilute aqueous solvent, to produce a solution of the substrate to which the amylase is added. In the case of the starch product prepared as described in chapter 4, preparation of a dilute aqueous solution is particularly hard and requires heating at high temperatures for extensive periods. If and when the material is finally dissolved the measured proportion of reducing ends is quite high. For this reasons the starch for this test was suspended in aqueous solution with dissolved amylase, without previous treatment. Amylase digestion resulted in a solution of sugars , however by the time a solution is produced the measured amount of reducing end groups is again high. This means that a sensible time-course could not be prepared for this product with points early in the treatment and for lower degrees of hydrolysis. However as seen in Figure 8.3, if a suspension of the starch ester is prepared in water and treated with an amount of hydrolysing enzyme, the overall hydrolysis (measured as equivalent reducing power of glucose present) is not as extensive as on a suspension of native untreated starch.

Tapioca starch as well as its decanoic acid ester were treated with 10 Units of α-amylase, for 2.5 hr. The graph clearly shows that the presence of ester groups on the starch inhibit the digestive action of the enzyme. It is obvious that even low substitutions have important effects. In our case the presence of one acyl group every one hundred anhydroglucose units, reduces the digestion by 11%.

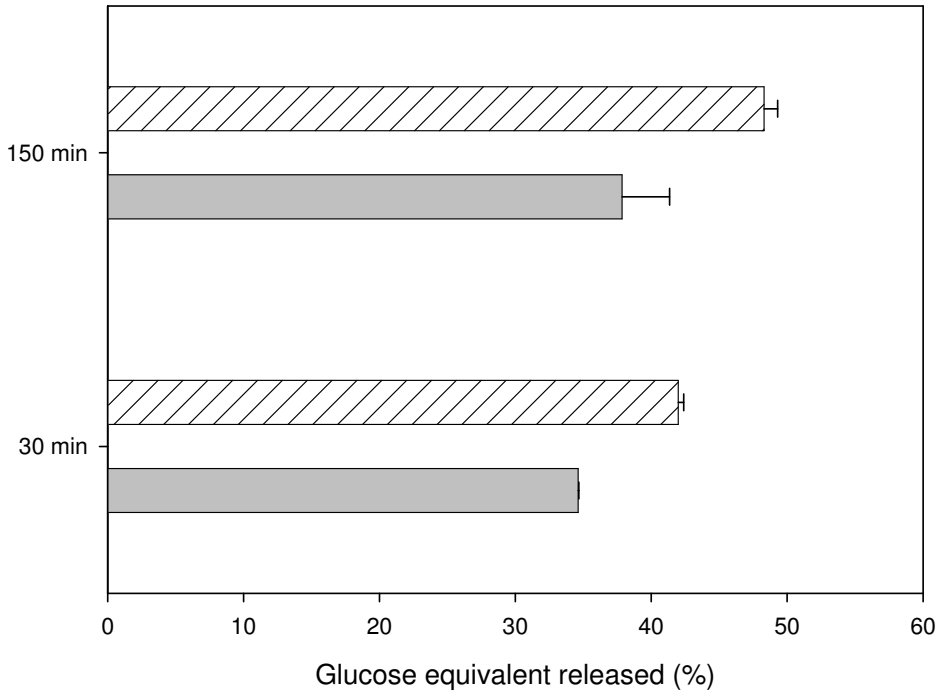

Figure 8.3 Glucose equivalent present in digestion mixture, after treatment with 1 units of α-amylase from *Aspergillus oryzae* per mg of starch, for native tapioca (striped bars) and 10-undecynoic acid tapioca ester (shadowed bars, DS=0.01). Errors are the standard deviation for experiments performed in triplicate.

8.3.2 α-Amylase digestion of starch ester prepared by enzymatic Amano A catalysis in [EMIM][DCA]

In the case of starch esters the product's physical appearance resembles that of the unmodified starch. It also can be dissolved in dilute aqueous solution by gelatinisation treatment. For this reason in the case of this product, the starch sample was heated for 1 hour at 90^{o}C until a clear aqueous solution was observed. This was then cooled and used as the starch sample in the amylase assay. In Figure 8.4 it is obvious that the acylation sites present in the ester inhibit the action of the hydrolytic enzyme.

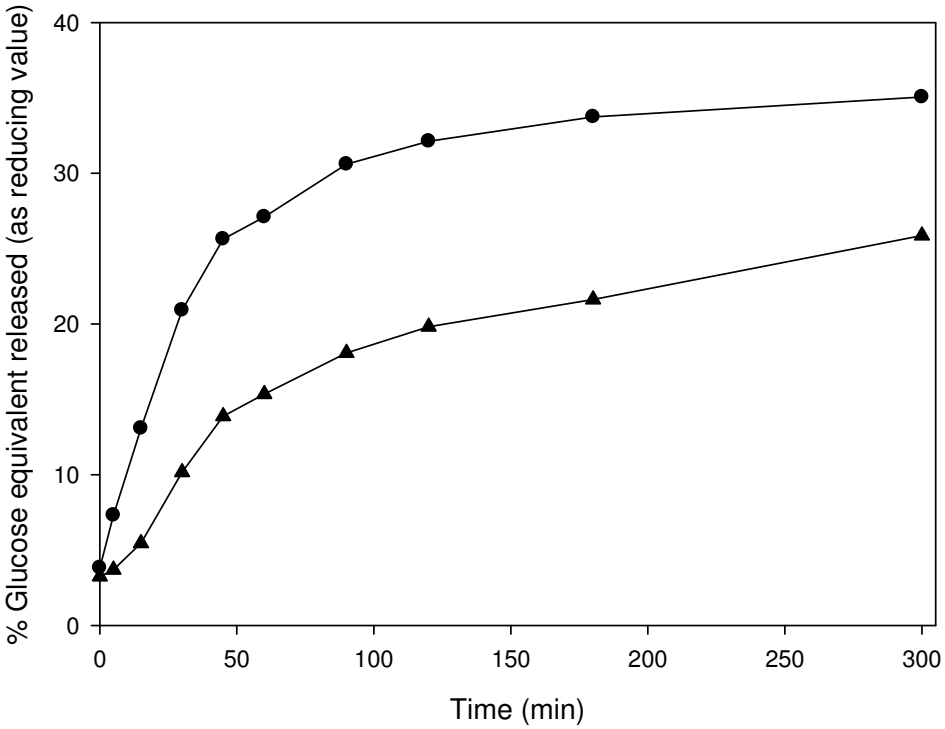

Figure 8.4 Hydrolysis of tapioca starch (●) and 10-undecynoic acid tapioca starch ester (DS 0.01) (▲), treated with 0.1 unit of α-amylase from *Aspergillus oryzae* per mg of starch.

8.3.3 Photograph microscopy of starch esters

Observations were made for unmodified native tapioca starch, gelatinised tapioca starch and tapioca starch esters of 10-undecynoic acid prepared in aqueous (chapter 5) and [EMIM][DCA] (chapter 7) reaction mixtures. The starch particles were dry prior to suspension in water for observation.

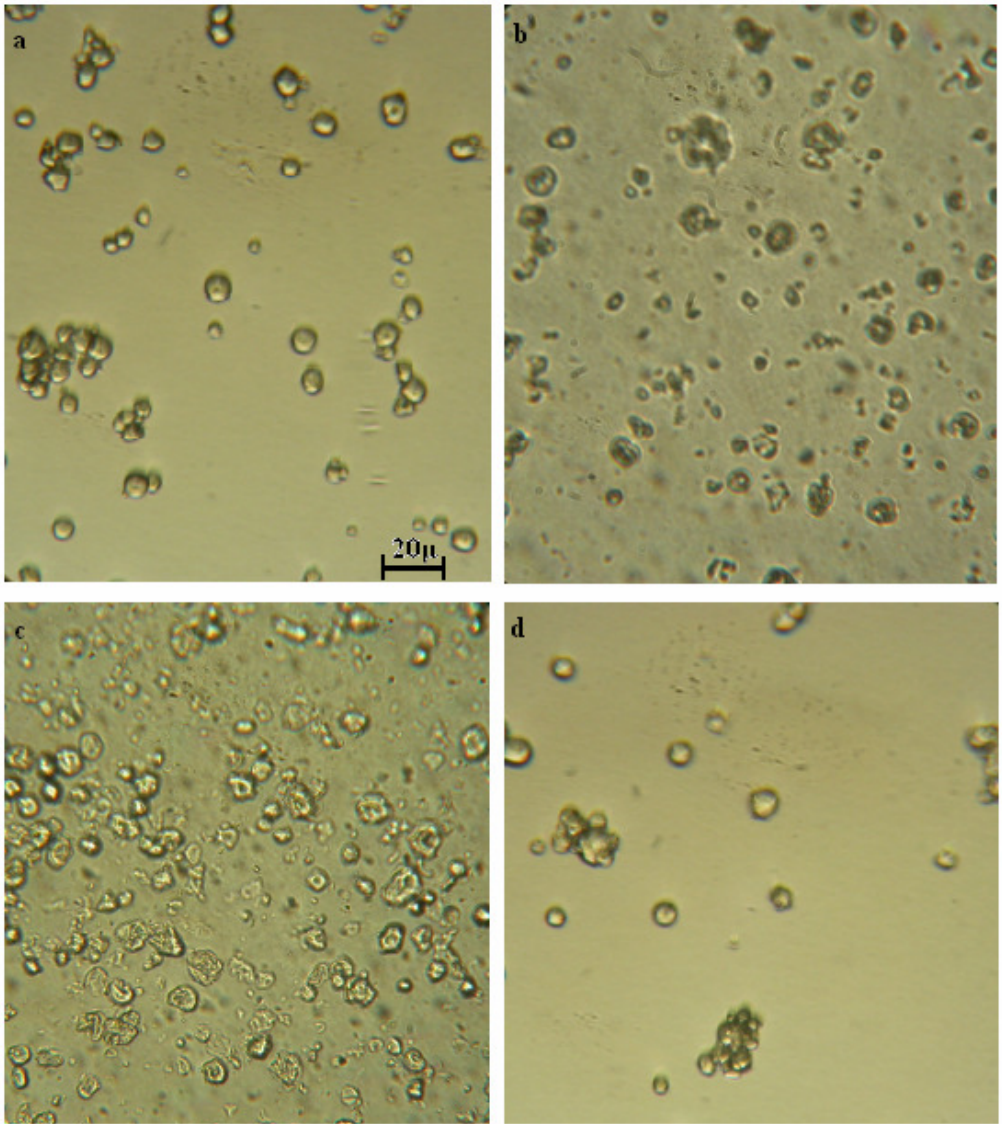

Figure 8.5 Photographs taken at x400 magnification for a. Tapioca starch, b. Gelatinised tapioca starch, c. Tapioca ester prepared in aqueous gelatinised mixture, d. Tapioca ester prepared in [EMIM][DCA]

In Figure 8.5c it is obvious that the starch esters produced in the aqueous gelatinised starch reaction mixture have lost granular structure. This is expected since the initial step, required for the reaction to proceed (chapter 4.3.5), is gelatinisation of the starch in water. This presumably releases the starch molecules from the granule making them more susceptible to enzymatic attack.

It is interesting however that this is not the case for the product of the [EMIM][DCA] reaction system. The starch in this case retains the granular structure to a great extent as can been seen in Figure 8.5d. Since the tapioca starch granules are approximately 1-20 μm in width, the retention of this structure throughout reaction implies that the starch is not dissolved in the ionic liquid, but rather very well dispersed. This is not surprising since no steps were taken to ensure dissolution before the reaction and the mixture of starch and IL actually was not a clear solution. Also the solubility of amylose in $[DCA]^-$ RTILs in literature (Liu *et al.*, 2005) is lower than the amount of starch used in the reactions described (chapter 7). However this also shows that the product of this reaction is actually different to the product of the aqueous reaction. Finally it can be perceived that treatment of the starch with the ionic liquid that may potentially lead to true dissolution (e.g. heating), could result in production of esters with higher DS values.

8.3.4 Observations on solubility

Although the solubility of the starch products prepared was not meticulously studied, certain observations could be made during the workup of experiments performed. The starch esters prepared from gelatinised starch reactions, showed increased resistance to "dissolution" in water even under conditions of dilute gelatinisation, where unmodified starch forms sols. This may be a result of elevated hydrophobicity of the acylated starches. However the non-enzymatic control starches showed similar behaviour, ergo it is more likely that it is a result of the properties of particles formed during the gelatinisation-precipitation routine. Furthermore these esters showed increased solubility in DMSO compared to the unmodified starches (and the controls). This could be the result of disturbance of non-covalent interactions between starch chains, due to the presence of the long acyl chains. This makes it easier for the solvent to penetrate the space between starch chains. These differences

in solubility could not be observed in the esters prepared in ionic liquid, probably because, as shown, the granular structure is still intact in this case.

8.4 Conclusions

Inhibition of α-amylase activity on starch fatty acid esters produced by lipase catalysed acylation was investigated. Starch ester produced by enzymatic esterification of gelatinised starch in concentrated aqueous gels, as described in chapter 4, presented difficulties in preparation of a homogeneous aqueous sample for digestion. For this reason the suspended starch was treated with amylase and the hydrolysis was measured after production of a clear solution. The starch ester produced by enzymatic means in ionic liquid [EMIM][DCA] on the other hand, could be prepared into a clear solution which was treated with α-amylase. In both cases an obvious inhibition of the enzymatic action was observed and the degree of hydrolysis ,measured as reducing equivalent of glucose present, was considerably lower than that observed in native starch, after hydrolysis reached a plateau. This suggests that even a small degree of acylation is sufficient to inhibit enzymatic degradation of the polysaccharide to a considerable extent.

Observation of the enzymatic reaction products prepared as described in chapters 5 and 7, with the aid of light microscopy led to some interesting observations. Starch esters prepared in the aqueous systems showed loss of granular structure as expected. However starch products of the ionic liquid [EMIM][DCA] system retained granular structure after the reaction.

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APPENDIX

A. Attempted protease catalysed acylation with α-aminoacids

Proteases are hydrolytic enzymes, that naturally conduct proteolysis, that is the hydrolysis of peptide bonds in polypeptide chains. Due to the commercial availability of proteases at reasonable prices and their stability, they have been popular candidates for catalysing synthetic reactions. Generally though, in the case of esterification or transesterification reactions they have been overshadowed by lipases and esterases, that are much more flexible in terms of substrate specificity. The strong substrate specificity of proteases limits their use to acyl groups that are closely related to their natural substrates. However there are some instances where the stability and specificity of proteases can make them the best candidate, such as enantiomeric resolutions of carboxylic acids or stereospecific acylation reactions. Subtilisin is usually the protease employed for acylation reactions, commonly in organic solvent systems where the absence of water drives the reaction towards synthesis of esters. There have been examples of ester syntheses of alcohols or even sugars with fatty acids (Liu & Tam, 2001; Weignerova *et al.*, 1999) and alcohols with aminoacids(Riva *et al.*, 1998). The protease catalysed synthesis of esters of carbohydrates and amino acids or oligopeptides has been investigated with two different approaches. The first was by catalysis of a peptide bond formation between amino acid and glycopeptide molecules(Wong *et al.*, 1993), the second is the direct acylation of carbohydrates by amino acids. Klibanov and co-workers catalysed the reaction of N-protected chloroethyl esters of amino acids with carbohydrates, with subtilisin in DMF, a very hydrophilic organic solvent (Riva *et al.*, 1988). Park et al. (Park *et al.*, 1999) synthesised similar molecules for disaccharides, in pyridine, using a commercial preparation of subtilisin. The use of free amino acids as acyl donors was reported by Lohith *et al.*(Lohith & Divakar, 2007), for the synthesis of aminoacyl esters of carbohydrates in a mixture of dichloromethane and DMF. However the enzyme used in this case was a lipase.

Experiments

Attempts were made to produce starch amino acid esters, with the help of proteases as biocatalysts. α-Chymotrypsin and subtilisin were both employed, in order to

esterify tyrosine, tryptophan or phenylalanine derivatives with the free hydroxyl groups in the glucan chain. Free L-tyrosine, the acetyl protected derivative (N-Acetyl Tyrosine) as well as the protected ethyl ester (Ac-Tyr-OEt), were used as substrates. The acetyl protected ethyl esters of phenylalanine (Ac-Phe-OEt) and tryptophan (Ac-Trp-OEt) were also tested. The systems employed were aqueous, where proteases have been known to show some favour towards alcohols as nucleophiles. One system was an excess of buffer, the other was gelatinised starch (40% buffer) and a third consisted of gelatinised starch but where an amount of buffer is added post gelatinisation and prior to the reaction. Systems for several organic solvents were also tested, such as pyridine, DMSO and DMF/dichloromethane. In all cases though, the proteases failed to catalyse the production of starch esters. The analysis was done after alkaline transesterification of the acyl donor (amino acid) to methanol and analysis of the methyl ester by HPLC-UV. Aromatic amino acids such as tyrosine, tryptophan and phenylalanine will absorb light at around 280 nm. Standards of the different amino acid derivatives were ran to measure their retention time for the specific HPLC method. In the case where ethyl esters were used, a methyl ester peak was detected, but was also present for the non enzymatic controls. This means that the likeliest source for the formation is residual ethyl ester, rather than starch ester.

Conclusions

The protease catalysed esterification or transesterification of starch with amino acid derivatives was attempted, but gave no product for the reaction systems that followed from the aqueous systems in which lipases successfully catalysed the synthesis of fatty acid esters of starch. Proteases also failed to catalyse ester formation in a number of organic solvent systems tested.

B. Attempted acylation with fatty acids, substituted in the alkyl chain

The relative promiscuity of lipases can be manipulated to achieve catalysis of reactions with fatty acids with substituted alkyl chains. In some cases the presence of other groups on the fatty acid chain will not inhibit access to the active site. A range of fatty acid related molecules were investigated as potential substrates for the lipase catalysed esterification of starch in aqueous gelatinised starch (chapter 4), in order to synthesise different types of starch esters. Namely ω-aminohexanoic acid, ω-aminodecanoic acid, adipic acid and 10-bromodecanoic acid were investigated.

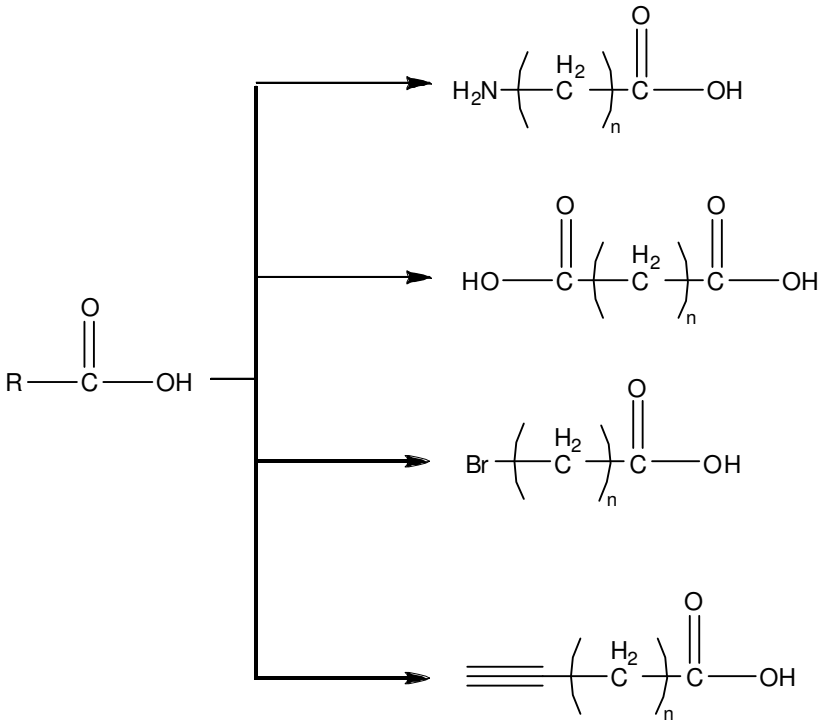

Figure B.1 Fatty acids with substitutions in the alkyl chain are commercially available, ω-aminoacids, dicarboxylic acids and halogenated acids were tested. ω-Triple bond unsaturated acids are described in chapter 5.

In all cases the presence of ester was investigated with the methanolysis/GC scheme (chapter 2). For the aminoacids, no ester formation could be detected. When adipic acid was employed as acyl donor and the product is transesterified in methanol for GC analysis, there are two possible analytes. The two carboxyl groups present, provide two potential sites for esterification with the nucleophile. Depending on this

mono- or di-methyl adipate, could be produced during methanolysis. After reaction and analysis, a peak was detected that matched mono-methyl adipate, suggesting the production of mono-esters of starch with adipic acid. However the DS obtained was very low (>0.001). It is noteworthy that after transesterification, the aqueous phase had to be acidified for the monomethyl adipate to extract to the organic phase for which diethyl ether was found to be appropriate (n-heptane was immiscible with monomethyl adipate).

Bromo-decanoic acid was also used as acyl donor. In this case after methanolysis and GC analysis, there were three detectable peaks that could not be identified. The presence of the bromo-group in the alkaline transesterification mixture could result in the formation of other methyl ester derivatives from a possible starch ester of 10-bromodecanoic acid. The presence of the halide could lead to alkali catalysed elimination or substitution, resulting in methyl esters of 10-decenoic acid, 10-hydroxydecanoic acid or 10-methoxydecanoic acid, in addition to the expected methyl 10-bromodecanoate. This would explain the detection of multiple peaks. The presence of the characteristic peaks for the two bromine isotopes could not be detected by GC-MS. In this case the estimated substitution is particularly low, while halogen containing esters of starch have been produced non-enzymatically but attracted no interest in terms of properties (Jarowenko, 1986; Tomasik & Schilling, 2004).

Conclusions

Investigation of potential use of molecules that may be recognised as substrates by the lipase for esterification of starch, resulted in formation of no ester (ω-aminoacids), very low substitution (dicarboxylic acid) or inconclusive results (bromoacid).

C. Solubilised enzyme

Bruno *et al.* (Bruno *et al.*, 1995), reported the use of solubilised enzyme as a catalyst for the acylation of undissolved amylose in non-polar solvent. In this technique an enzymatic solution is added to a water immiscible organic solvent (e.g. isooctane) containing ionic surfactant in smaller amounts than required to completely coat the enzyme surface. A clear organic phase is obtained in which the enzyme is dispersed, while remaining active due to the action of the surfactant. The authors note that there is not sufficient surfactant to form reversed micelles, suggesting that the molecules form ion-pairs with specific enzymatic sites instead, thus protecting the enzyme from inactivation (Wangikar *et al.*, 1997).

This technique was attempted for the enzyme that is known to catalyse the acylation of starch with fatty acid (chapter 1), lipase from *Thermomyces lanuginosus*. 5 mL of lipolase (5 kU) in aqueous buffer (pH=7.0 potassium acetate 10 mM with 10 mM potassium chloride and 10 mM calcium chloride) were added dropwise to 5 mL of anionic surfactant Aerosol OT solution in *n*-heptane (25 mM), with magnetic stirring. During the mixing step, the presence of an emulsification layer between the aqueous and organic phases, could not be avoided. Furthermore formation of precipitated lipase could be visually detected. The mixture was left to mix at room temperature overnight. The organic phase was then retrieved and its absorbance scanned in a UV-Vis spectrophotometer. No peak could be detected at 280 nm, characteristic for proteins (due to aminoacids with aromatic rings). The unsuccessful solubilisation of lipolase was confirmed by the lack of acylation when the enzyme in the organic solvent was co-incubated with dry starch and decanoic acid or vinyl decanoate. Use of other buffers, *n*-hexane and changing the AOT concentration did not result in successful solubilisation.

Conclusions

Lipolase, known to catalyse esterification of starch with fatty acid in gelatinised starch aqueous systems (chapter 1), could not be successfully solubilised in organic solvent in order to carry out enzymatic acylation of suspended starch in said solvent.

D. References

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OVERVIEW

The experiments described throughout the present thesis have yielded some extremely interesting results for both the polysaccharide and biocatalysis scientific communities.

The need for a fast and reliable product analysis method led to the investigation of available DS quantification methods, revealing shortcomings of some well established approaches. These need to be taken into consideration in similar future investigations, regardless of the starch acylation technique employed. The properties of the acylated starch depend greatly on the DS. A lightly substituted product will be totally different to a fully acylated starch, so the appropriate analytical tool may vary with substitution. However the scheme suggested in the present study, i.e. combining acylated starch alkaline methanolysis with GC-FID of the resulting methyl esters, was a convenient and accurate method for the analysis of the low DS products synthesised. Furthermore, there is no apparent reason for the scheme not to be applicable, with the same success, to acylated starches of any DS. The main advantage of this approach is that it distinguishes between true ester and residual carboxylic acid substrate, since only the transesterification to methanol takes place in the conditions employed. This is not easily done at low DS values with the likes of NMR and FTIR and impossible with the titration method, leading to the requirement for starch ester completely devoid of unreacted carboxylic acid (very difficult for fatty acid-type acyl donors). The only disadvantage is that it is an indirect method, unlike NMR and FTIR where the starch ester is directly analysed. These results will also interest investigators of polysaccharide modification in general, since in many cases the techniques are identical for different glycan esters.

The potential of an enzymatically catalysed starch acylation process was approached through several different routes. A possible system can be varied in relation to, amongst other parameters, the physical state of the ingredients (e.g. solute, liquid, solid or gel), the form of the enzymatic preparation (solution, suspended powder, immobilised) as well as the presence of water. This study concluded that the lipase catalysed acylation of starch with fatty acid is possible in the appropriate reaction medium. However none of the attempted reactions proceeded with suspended granular starch. The polysaccharide had to be either pretreated or dissolved, presumably thus minimising steric hindrance of enzyme action. The gelatinised

starch system (chapters 4 and 5) is particularly interesting, as water is the only compound present in the reaction mixture, apart from the reactants and catalyst. This is an added bonus to a process which is already benign due to the enzymatic nature of the catalysis. This system is also interesting from an enzyme chemist's perspective due to its unusual configuration. Enzymes have been found to remain active in many types of low-water media and a system where one reactant is in aqueous gel form and acts as the dispersant for the other substrate is an intriguing addition to this list. The use of ionic liquids as reaction media, due to their potential for polysaccharide dissolution, was also investigated. Ionic liquids have attracted much attention in biocatalysis, as an alternative system with low water content, that may dissolve a range of compounds with significantly different polarities. In this way polar and non-polar substrates can coexist in a single liquid phase, facilitating the biotransformation. However it was observed that the ionic liquids that display this large potential as solvents, also inactivate the enzyme. This may be due to stripping of essential water molecules from the protein surface, or disruption of non-covalent bonding between aminoacids, leading to unfolding and loss of active conformation. Ionic liquids of more moderate dissolving potential that are still able to accommodate polysaccharides, appeared to be suitable for enzyme synthetic action. Enzymatic starch acylation in such solvents has not been reported in literature, however the difficulty in product separation and the formation of by-products, makes these systems very complex and accurate product characterisation extremely difficult.

An investigation into different substrates, that may be accepted as acyl-donors by the enzyme, led to the synthesis of a novel starch ester with great potential. The starch esters of ω-alkynoic acid have acyl groups extending from the polymer chain, with triple bonds at terminal positions. The strong reactivity of these with azide groups, under mild and diverse conditions, means that the acyl chains may act as linkers for attachment of any compound containing the azide moiety. This leads to a mild two-step reaction process for the synthesis of acylated starch, derivatised with a vast array of compounds.

Of course a study of such a general subject, for the scope of a PhD, can in no way be conclusive. The research conducted barely scratches the surface of the array of potential approaches to the matter. The intention has been to investigate those

approaches that seemed most likely to yield significant results. The systems that have been found appropriate for the synthesis of acylated starch, have by no means been exhaustively optimised. There still may be much room for improvement with aid of the principles of what has been termed "medium engineering". This will be facilitated by a better understanding of the processes governing the kinetics and thermodynamics of the complex heterogeneous systems described. In the case of the gelatinised system for example true reaction equilibrium may not have been reached under the conditions described, as there may be other limiting factors present. Furthermore, tools of molecular biologists, such as protein engineering, which is totally out with the scope of the present investigation, may be used to yield new enzymes. These may combine acylating activity and carbohydrate recognition, making them better equipped for catalysis of this reaction.

As mentioned, the chemical acylation of starch is a reaction that has been greatly researched in the past and many approaches to this process have been suggested. Despite the obvious advantage of the milder reaction conditions in enzyme catalysis, it is very likely that there are other benefits that have not been proved in the present investigation. Hydrolases have been known to present strong selectivity towards the different hydroxyls present in carbohydrates, usually favouring the primary hydroxyl (though this is not always the case). Proof of this regioselectivity in the enzymatic acylation of starch would differentiate it to the random chemical acylation. Also investigation of the acylation pattern on the polymer chain could lead to interesting observations and display that the enzymatic product is novel in this respect also.

Finally further research into the possibility of alkyne/azide cycloaddition on the triple bond of the ω-alkynoic acid starch esters, will display the ability of this acyl groups to operate as linkers. This reaction is known to proceed in a wide range of solvents (e.g. water, DMSO, acetonitrile), under mild conditions. Investigation of the reaction conditions necessary to perform such an addition on the starch ester will open new routes towards a potentially vast array of starch derivatives, since many compounds functionalised with the azide group have become commercially available.

In conclusion this study provides an initial understanding of the potential of different approaches to the enzymatic acylation of starch. It has displayed the feasibility of such a reaction, despite obvious hindering factors, and described the synthesis of a

novel starch ester, displaying yet again how biocatalysis can be used in conjuction with other synthetic tools to yield products of great potential.