CHALMERS

Biocatalysis in Different Polymer Solutions

Biotransformations with trypsin in solutions of polymers with different charges

Master of Science Thesis in the Master Degree Program, Biotechnology

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Applied Surface Chemistry

CHALMERS UNIVERSITY OF TECHNOLOGY

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Abstract

Previous experiments conducted at the department of Applied Surface Chemistry at Chalmers with the enzyme trypsin in an alginate gel showed activation of the biotransformation by the inhibitor D-BAPA. The purpose of this study was to further investigate the cause and generality of the observed activation by D-BAPA. In the study biocatalysis was conducted in solutions of polymers of different charges as well as in other alginate products. No similar activation by D-BAPA could be found in any of the experiments. Instead a change from the cationic substrate L-BAPA to a zwitterionic substrate was made. Results indicate that the effect could be partly caused by interactions of BAPA and the polymer. Another possibility is that the effect is caused by a conformational change of the enzyme; however this was not possible to study.

Keywords: trypsin, biocatalysis, polymer solution, alginate, L-BAPA, D-BAPA, uv-vis spectroscopy

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1. Introduction

Previous experiments conducted at the department of Applied Surface Chemistry at Chalmers with the enzyme trypsin immobilized in an alginate gel gave unpredicted results. Biotransformation of the substrate Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPA) was performed in presence of an inhibitor Nα-benzoyl-D-arginine 4-nitroanilide hydrochloride (D-BAPA), which is the enantiomer of the substrate. At 37°C in buffer solution D-BAPA acts as an inhibitor thus lowering the activity of the enzyme. However when the same experiment was conducted in alginate gel activation of the reaction was observed.

One proposed explanation to this unpredicted behavior is interactions of the enzyme with the alginate polymer. As the alginate polymer at the studied pH has a negative charge and the bovine trypsin a positive charge electrostatic binding effects could be at play. Another possibility is that something in the structure of the alginate apart from charge produces a change in the conformation of the enzyme. The effect could also be caused by binding effects between the BAPA and the alginate.

In order to investigate the cause of the activation by D-BAPA biocatalysis in different polymer solutions of varying charge and structure was performed. Experiments were also made in solutions of other alginate products. Biotransformation with a substrate of other ionic charge was also conducted.

1.1 Objective

The purpose of this study was to further investigate the cause and generality of the observed activation by D-BAPA.

2. Background

In this section some background information is given. A short introduction to enzyme kinetics is provided which is followed by information about trypsin and substrates and inhibitor used in the study. The next section gives descriptions of all different polymers and some of their previous applications in enzyme technology. The last section is dedicated to instrumental techniques.

2.1 Introduction to enzyme kinetics

Enzymes are biocatalysts that convert a substrate into a product. Enzymes increase the rate of the chemical reaction by stabilizing the transition state of the reaction and thus lowering the activation energy of the reaction. In general, enzymes are highly specific and only convert specific substrates into product. An enzymatic reaction where a substrate is converted into a product can be described by this simplified reaction scheme;

$$\mathrm{E} + \mathrm{S} \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_{\mathrm{cat}}} \mathrm{E} + \mathrm{P}$$

In the above schematic of the reaction E signifies the enzyme, S the substrate and P the product. The reaction is essentially divided into two steps, the formation of a binary complex of substrate and enzyme and then formation of product. The parameter K_s is the equilibrium dissociation constant and is defined as the ratio of K_{off}/K_{on}. The constant K_{cat} is a value that describes how rapidly product is formed. The reaction has pseudo-first-order kinetics (presuming that there is initially a much higher concentration of substrate than enzyme). The reaction proceeds until all substrate is consumed. In the very beginning of reaction the formation of product is linear. The initial speed v can thus be determined as the slope of the line when plotting increasing product concentration as a function of time. The initial speed of the reaction is dependent on enzyme and substrate complex and on K_{cat} according to; $v=K_{cat}[ES]$

An important equation in enzyme kinetics is the Michaelis-Menten equation:

$$\frac{\qquad}{\quad}$$

The constant K_m is a measure of the affinity with which the substrate binds to the enzyme. Low values of K_m indicate strong binding. The initial speed v increases with increasing substrate concentration over a finite range. By plotting v as a function of substrate concentration K_m and V_{max} can be determined. K_{cat} can then be determined by diving V_{max} by [E]. For a longer introduction to enzyme kinetics and the determination of kinetic constants see (Copeland 2001).

2.2 Trypsin

Trypsin is a digestive enzyme that is present in the intestine of vertebrate animals. It cleaves peptide bonds by hydrolysis after Lysine or Arginine residues. The bond enters the active site where the transition state is stabilized by catalytic residues. Hydrolysis only occurs at peptide bonds consisting of L-amino acids as peptide bonds of D-amino acids are misaligned in the active site.(Hedstrom 2001)

As trypsin cleaves peptides the enzyme is subjected to autodigestion; a process that is proportional to the enzyme concentration.

Refolding of denatured trypsin has been carried out in some studies. This was done by first denaturing trypsin using concentrated guanidinium chloride and then diluting the enzyme and denaturing agent in a media. In one study the use of diethanolamine as refolding media proved to

increase recovered activity of the enzyme. (Ohshima, Suzuki et al. 2008) In some studies inhibitor of trypsin has been used to avoid autodigestion in refolding processes. (Nohara, Kondo et al. 2004) In one study the refolding yield was increased by 20 % by using an inhibitor. (Nohara, Sugiura et al. 1999)

D-BAPA and L-BAPA

Nα -benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPA) is a synthetic substrate for trypsin, when L-BAPA is cleaved para-nitroaniline is formed and its absorbance can be measured by UV-vis spectroscopy at 405 nm. Nα-benzoyl-D-arginine 4-nitroanilide hydrochloride (D-BAPA) is the enantiomer of L-BAPA and is a competitive inhibitor of trypsin. The structures of D-BAPA and L-BAPA are shown in Figure 1.

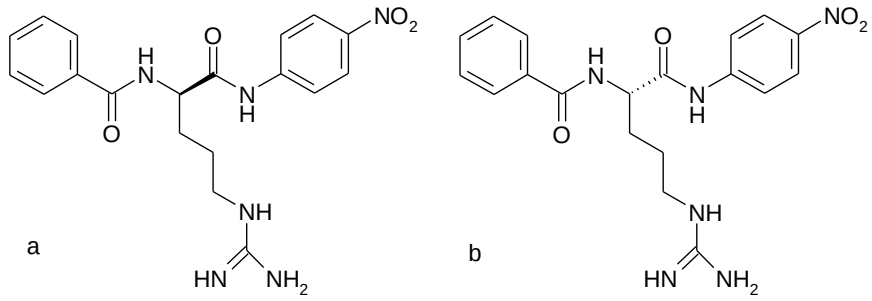

Figure 1. The structure of a: Nα-benzoyl-D-arginine 4-nitroanilide hydrochloride (D-BAPA) and b: Nα -benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPA).

L-ZAPA

*N*α–benzyloxycarbonyl–L–arginine–*p* nitroanilide hydrochloride (L-ZAPA) is a synthetic substrate for trypsin very similar in structure to L-BAPA. Upon cleavage para-nitroaniline is formed and its absorbance can be measured by UV-vis spectroscopy at 405 nm. The structure of L-ZAPA is shown in Figure 2.

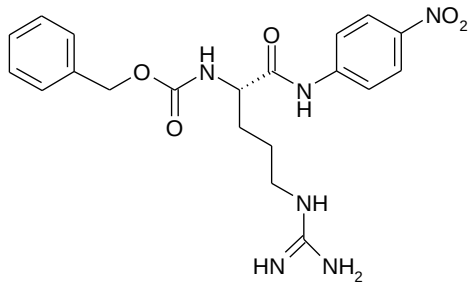

Figure 2. The chemical structure of Nα-benzyloxycarbonyl-L-arginine-p nitroanilide hydrochloride (L-ZAPA)

Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide

Another substrate for trypsin is Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide that also produces para-nitroaniline upon cleavage. This substrate contains a negatively charged deprotonated carboxylate group at pH 7.6. The structure is shown in Figure 3.

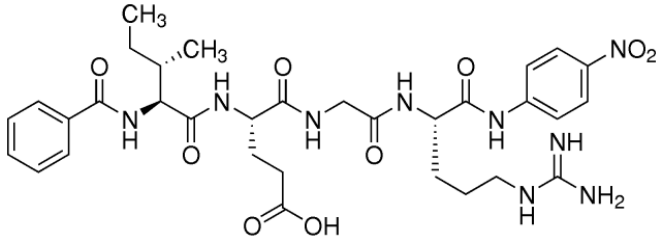

Figure 3. Structure of Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide.

2.3 Polymers

In this study aqueous solutions of different polymers were used for biocatalysis with trypsin. The polymers were chosen because of their different structures, molecular weights and ionic charges at pH 7.6. Some of the polymers are previously known to interact with proteins. Below is an introduction to the polymers and some of their previous applications in enzyme and protein technology.

2.3.1 Alginate

Alginate is a biopolymer produced by brown seaweed and some bacterial species. It is commonly used in the food industry and in the pharmaceutical industry because of its gelling, stabilizing and thickening properties. Alginates are linear polysaccharides that consist of 1->4 linked β-D-Mannuronic acid and α-L-Guluronic acid. The polymer consists of homopolymeric blocks of β-D-Mannuronic acid and α-L-Guluronic acid as well as blocks with approximately alternating residues.(Arne Haug 1967) The composition of the polymer is highly variable and depends on factors such as season for harvest and type of algae.(Black 1950; Hans Grasdalen 1979)

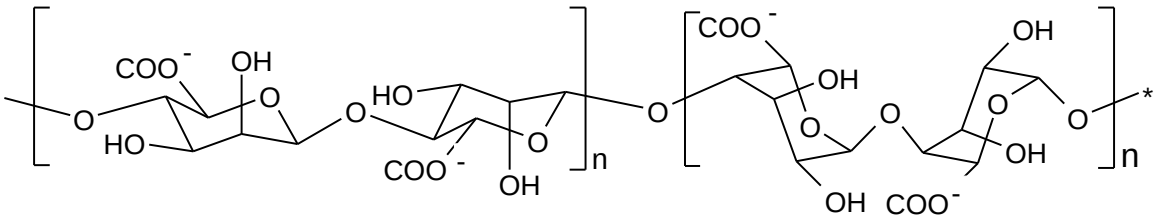

Figure 4. Structure of alginate, to the left mannuronic acid block and to the right guluronic acid block. Alginate also contains blocks with alternating mannuronic and guluronic residues (not shown).

The structure of alginate is given in Figure 4. The chair conformation of the residues (see Figure 4) has been determined by C-NMR spectroscopy (Grasdalen, Larsen et al. 1977) and H-NMR spectroscopy (Penman and Sanderson 1972).

Alginate and its interactions with enzymes have previously been examined in different studies. Recently chymotrypsin was purified from pancreas homogenate by adsorption onto alginate beads. (Spelzini, Farruggia et al. 2011) The same study also measured trypsin activity in the purified samples and it was found that some purification of trypsin also had occurred. Purification of different lipases by affinity precipitation has been performed with alginate giving yields of up to 87 %. In the same study it was found that alginate activates wheat germ lipase up to 5 fold. Protection of the enzyme by alginate against thermoinactivation was also determined.(Sharma and Gupta 2001) Alginate with high guluronic acid content has been used to refold lipase, after refolding the enzyme could be recovered by affinity precipitation. (Mondal, Bohidar et al. 2006) In a study purification of pectinase from *Aspergillus niger* by precipitation of alginate was found to be increased by microwave pretreatment of the polymer.(Mondal, Mehta et al. 2004)

2.3.2 Polyethyleneimine

Polyethyleneimine (PEI) is a synthetic polymer. The structure is shown in Figure 5. Polyethyleneimine has been used extensively for immobilization of enzymes. (Bahulekar, Ayyangar et al. 1991)

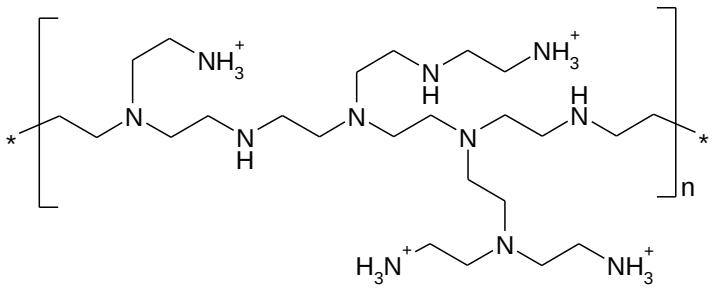

Figure 5. Structure of polyethyleneimine showing some possible protonation of nitrogens.

PEI has been shown to have a stabilizing effect on trypsin and other proteins (Andersson and Hatti-Kaul 1999)

2.3.3 Polyethylene glycol

Polyethylene glycols (PEGs) are used in the cosmetics industry and pharmaceutical industry. Some examples of use are in creams and as tablet and pill binders. The structure of PEG is shown below in Figure 6.

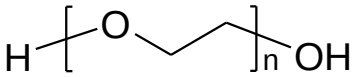

Figure 6. Structure of polyethylene glycol.

PEG has previously been used in studies of enzymes; as an example it has been shown to significantly reduce inactivation of the enzyme laccase (Modaressi, Taylor et al. 2005) In another study trypsin-PEG conjugates were made and it was found that conjugates with a PEG molecular weight of 5000 Daltons had better stability than native trypsin. (Treetharnmathurot, Ovartlarnporn et al. 2008) A similar study found that trypsin-PEG conjugates had both improved thermal stability, resistance to detergents and higher activity.(Gaertner and Puigserver 1992)

2.3.4 Polyacrylic acid

Polyacrylic acid (PAA) is an anionic polymer. It is deprotonated at physiological pH. The structure is shown in Figure 7.

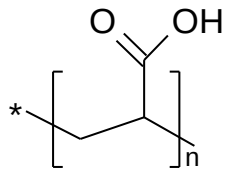

Figure 7. The structure of polyacrylic acid.

PAA has been shown to inhibit trypsin activity and its possibilities for delivery of peptide drugs have been assessed (Ameye, Voorspoels et al. 2001)). The reason for the inhibition is believed to partly be caused by formation of an ion-complex between the polymer and the substrate used N-a-benzoyl-L-arginine-ethylester. The structure of N-a-benzoyl-L-arginine-ethylester is shown in appendix for the interested reader. The inhibition is negligible at higher substrate concentrations.(Ameye, Voorspoels et al. 2000)

2.3.5 Polyvinylsulfuric acid

Polyvinylsulfuric acid is a synthetic polymer and the structure is shown in Figure 8.

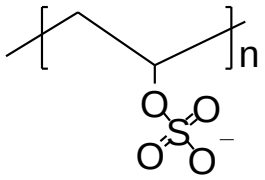

Figure 8. Structure of polyvinylsulfuric acid.

2.3.6 Xanthan

Xanthan is a biopolymer used in food-, cosmetic - and pharmaceutical industry where it is often used as a thickener or a stabilizer. It is an extracellular polysaccharide produced by the bacteria *Xanthomonas Campestris.* The structure consists of repeating units with 5 sugar residues; two glucose, two mannose and one glucuronic acid. The backbone of xanthan is formed by 1, 4-linked ß-D-glucose. (Jansson, Kenne et al. 1975; Melton, Mindt et al. 1976) The structure can be seen in Figure 9. In solution the conformation of xanthan has been proposed to be rod-like with some flexibility. (Whitcomb and Macosko 1978)

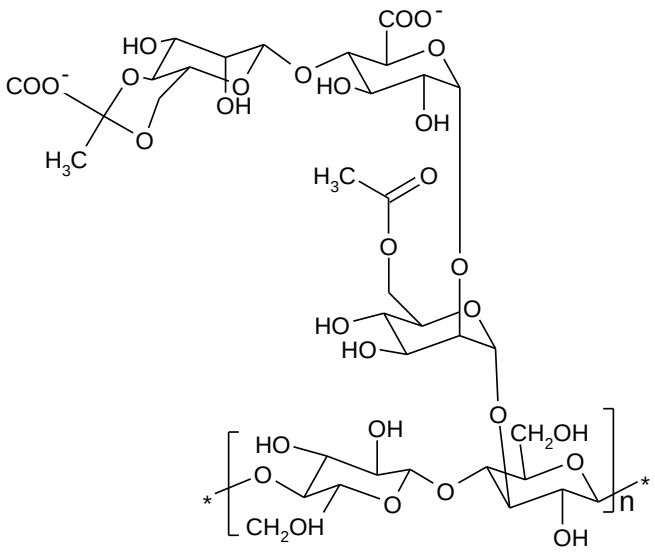

Figure 9. Structure of Xanthan.

In a previous study the enzyme urease was encapsulated in xanthan-alginate spheres resulting in improved stability of the enzyme.(Elçin 1995)

2.3.7 Carboxymethyl cellulose

Carboxymethyl cellulose(CMC) is a cellulose derivative biopolymer. It is used in the food industry as a thickener and stabilizer. It is also used as a pharmaceutical ingredient. The structure of CMC is shown in Figure 10.

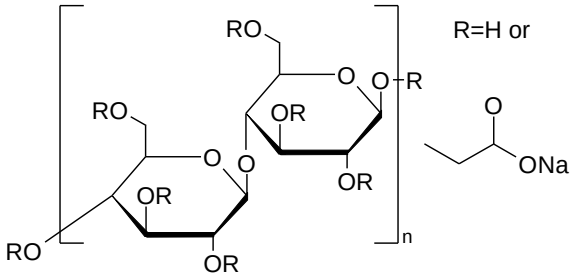

Figure 10. Structure of carboxymethylcellulose.

In a study trypsin was modified by a polyaldehyde derivative of CMC via reductive alkylation resulting in improved thermostability and resistance to denaturing agents.(Villalonga, Villalonga et al. 2000) In another study CMC was used to purify Lactoperoxidase from a model system by precipitation. (Lali, N et al. 2000)

2.4 Techniques

In this section a short introduction is given to the different techniques used in the study. UV-Vis Spectroscopy was used to measure product formation and fluorescence spectroscopy to study protein conformation.

2.4.1 UV-Vis Spectroscopy

UV-Visible Spectroscopy measures the interaction of molecules with electromagnetic radiation. The energy of light from the near-ultraviolet (UV) and visible (vis) range of the electromagnetic spectrum excites electrons from a ground state to an excited state. The intensity of the light decreases as it passes through the sample, the absorbance is dependent on the intensity of the light before and after the passage according to;

$$A=-log_{10}(I/I_0)$$

Where A is the absorbance, I is the intensity of the light after passage and I_0 is the intensity of the light before passing through the sample. The wavelength of absorbance depends on the molecule and also to some extent on its environment. Absorbance is linearly dependent on the concentration according to the Lambert-Beer law;

$$A=\varepsilon cl$$

Where c is the molar concentration, l is the pathlength, and ε is the molar absorption coefficient. (Schmid 2001)

Para-nitroaniline

When trypsin cleaves the substrates in this study para-nitroaniline is formed which absorbance can be measured at 405 nm. The molar absorption coefficient of para-nitroaniline is 10500 $M^{-1}cm^{-1}$ at pH 7.6.

2.4.2 Fluorescence Spectroscopy

Fluorophores are molecules that can absorb electromagnetic radiation at one wavelength and emit electromagnetic radiation at another wavelength. The fluorescence spectrum of a fluorophore depends on the environment it is in. The amino acids tryptophan and tyrosin are fluorescent which make them useful when studying proteins. Changes in the environment of a flurophore can be seen in the fluorescence spectrum which gives information about folding and structure of the protein. (So and Dong 2001)

3. Materials

This section lists all chemicals and other products used in the experimental work.

Alginates

Several different alginates were used in this study, all of them are sodium alginates derived from brown seaweed. Three different alginates of from FMC Biopolymer were used; Manugel DMB, Keltone® LVCR and Protanal® LF 10/60 LS. Also used were Pronova UP MVM and Pronova UP MVG both from Novamatrix™. An overview of the different alginates and their characteristics is given in Table 1.

Alginate product	Approximate Mannuronic acid content	Approximate Guluronic acid content	Approximate average molecular weight (Daltons)	Grade
Manugel DMB	30-40 %	60-70 %	150 000	Food
Keltone® LVCR	60 %	40 %	54 000	Food
Protanal® LF 10/60 LS	55-65 %	35-45 %	150 000	Food
Pronova UP MVM	51 %	49 %	200 000-300000	Analytical
Pronova UP MVG	30 %	70 %	200 000-300000	Analytical

Table 1. Overview of the different alginate products used in the study and their properties.

Polymers

Poly(ethyleneimine) solution of 50 % (w/v) in water was purchased from Sigma-Aldrich with an average molecular weight of 2000 Daltons. Poly(ethylene glycol) chips were purchased from Sigma-Aldrich with an average molecular weight of 2050 Daltons. Poly(acrylic acid) solution of 50 % (w/v) in water was acquired from Sigma-Aldrich with an average molecular weight of 2000 Dalton. Polyvinylsulfuric acid potassium salt was from Acros Organics with an average molecular weight of 175000 Daltons. The polymer xanthan used was the product Xantural® 180 from CP Kelco with an approximate average molecular weight of 2 million Daltons, pharmaceutical grade. Sodium carboxymethyl cellulose was purchased from Sigma-Aldrich with an average molecular weight of 250 000 Daltons. An overview of the different polymers and their characteristics is given in Table 2.

Polymer	Charge in solution with pH 7.6	Average molecular weight (Daltons)
Poly(ethyleneimine)	+	2000
Poly(ethylene glycol)	neutral	2050
Poly(acrylic acid)	-	2000
Polyvinylsulfuric acid	-	175000
xanthan	-	Approx. 2 000 000
carboxymethyl cellulose	-	Approx. 250 000
alginate	-	Approx. 54 000-300 000

Table 2. Overview of the different polymers and their characteristics.

Substrates, Inhibitor and Enzyme

Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPA), Nα-benzoyl-D-arginine 4-nitroanilide hydrochloride (D-BAPA), DL-BAPA with a ratio of D:L of 1:1 and *N*α−benzyloxycarbonyl−L−arginine−*p* nitroanilide hydrochloride (L-ZAPA) were all purchased from Bachem. Trypsin type I from bovine pancreas was from Sigma-Aldrich. Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide was also purchased from Sigma-Aldrich.

Other materials

The buffer used was biotechnology performance certified Trizma® base from Sigma-Aldrich. For denaturation of trypsin guanidine hydrochloride from Sigma-Aldrich was used. For dissolving substrates and inhibitor Dimethyl sulfoxide (DMSO) BioReagent from Sigma-Aldrich was purchased. For dissolving the substrate Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide Acetonitrile from Sigma-Aldrich was used. For purification of Manugel DMB by dialysis a membrane with a cut-off of 12000-14000 Daltons from Spectra/Por® was used.

4. Methods

In this section the experimental work is described.

4.1 General Procedures

In this study many different samples were made with a general procedure. For simplicity the general procedures are described in this section. If any sample preparation differs from standard procedure so will be indicated in the concerned section.

4.1.1 Sample Preparation

Each sample was prepared by mixing the following in a cuvette; 1.5 g of 2 % (w/v) polymer solution in buffer, 1.1 ml buffer and 200 µl of BAPA solution. There were two types of BAPA solution used, 2 mM L-BAPA in 5 % dimethylsulfoxide (DMSO) and 4 mM DL-BAPA in 5 % DMSO. The two samples will be referred to as L-BAPA and DL-BAPA samples throughout the study. The rest (95 %) of the solvent consisted of deionized water. Lastly 200 µl of 1.91 µM trypsin in 1 mM HCl was added to the samples.

The Tris buffer used had a concentration of 0.1 M and a pH of 7.6. For all buffer samples the polymer portion of the sample was replaced by buffer.

The final concentrations in the samples were of polymer 1 % (w/v), enzyme 127 nM, L-BAPA 133 µM, DL-BAPA 267 µM.

Control samples

Three types of control samples were used in the study. Control samples were made for both DL-BAPA and L-BAPA samples where the enzyme solution was replaced by 1 mM HCl. The third control type was made with 2 mM D-BAPA in 5 % dimethylsulfoxide (DMSO) and enzyme in order to verify that no transformation of the D-BAPA to product occurred. (Results from all control runs are shown in Appendix)

4.1.2 UV-Vis Spectroscopy

The absorbance at 405 nm was measured using a HP-8453 UV-vis spectrophotometer. The temperature was set using a water bath connected to the spectrophotometer. For the room temperature experiments the ambient temperature was used. All measurements were started without delay after addition of enzyme.

For the experiments where kinetics were studied over 15 hours the absorbance was recorded continuously every 10 minutes. For the experiments for determination of kinetic constants the absorbance was measured continuously every 30 seconds for up to 2 hours.

Handling of Absorbance Data

For all measurements with L-BAPA as substrate the initial absorbance of the sample was deducted so the start absorbance of all samples was equal to zero. Depending on the results from the control run the data was handled differently. How the control run data was used will be indicated in the concerned results section.

4.2 Catalysis in Different Polymer Solutions and Buffer

Catalysis in polymer solutions was conducted with 6 different polymers and also in buffer. The alginate product used in this part of the study was Manugel DMB. The experiments were repeated at three temperatures, 10°C, room temperature and 37°C. A schematic description of the experimental plan is shown in Figure 11.

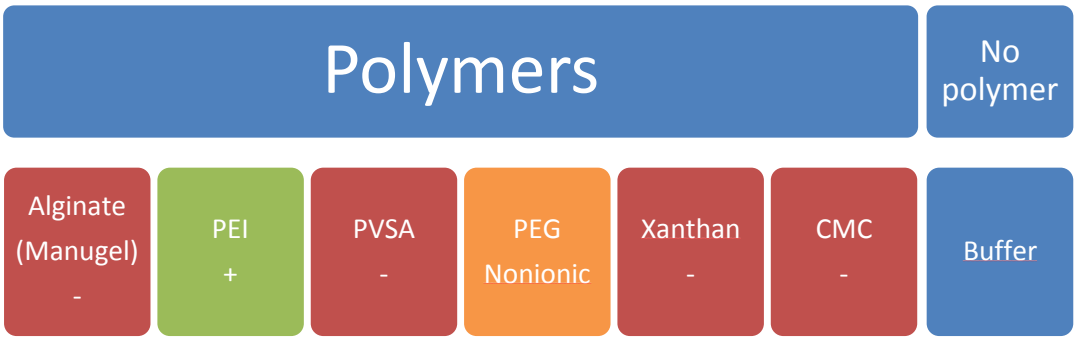

Figure 11. Illustration of the overall experimental design. Abbreviations are; PEI Polyethyleneimine, CMC carboxymethylcellulose, PVSA Polyvinylsulfuric acid, PEG polyethyleneglycol. The experiment was repeated at three temperatures 10°C, room temperature and 37°C. The signs + / – indicate the net charge of the polymer at pH 7.6.

Every experiment consisted of six samples with one polymer. Half of the samples were made with L-BAPA and half with DL-BAPA. Control samples were made in separate runs. All three types of control samples (see 4.1.1) were made in duplicates.

All runs were made once except for the run with Manugel at 37°C which was made twice.

Additionally one experiment was made with Polyacrylic acid at room temperature.

4.2.1 Catalysis in Solutions of Different Alginate Products

Catalysis with trypsin in alginate was further investigated by testing different alginate products at 37°C.

Ultrapure alginate products

Biocatalysis was done in two ultrapure alginates and buffer at 37°C. The ultrapure alginates are Pronova UP MVM . These alginates will be referred to as MVM and MVG throughout this report. Each experiment consisted of; two samples of MVM, two samples of MVG and two samples with buffer. One of each sample was made with L-BAPA and one with DL-BAPA. The experiment was replicated twice. An illustration of the experimental design is shown in Figure 12.

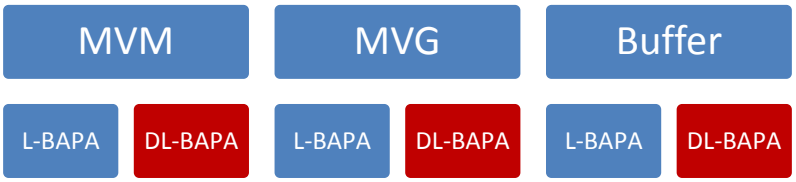

Figure 12. Schematic description of the experiment with trypsin catalysis in two ultrapure alginates, Pronova UP MVM and Pronova UP MVG. Buffer was also used for comparison. This experiment was made in two replicates.

Alginate products of food grade

Catalysis was also conducted in two other alginates at 37°C, Protanal and Kelton. The experimental setup is shown schematically below in Figure 13.

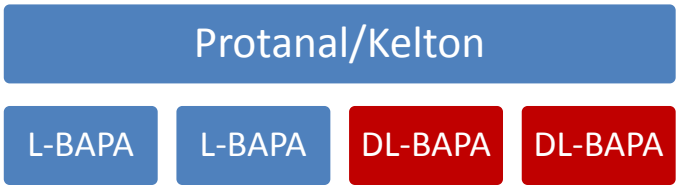

Figure 13. Schematic description of the samples in an experiment with pharmaceutical grade alginates. The experiment was conducted twice, once with Protanal and once with Kelton. There were four samples; two with DL-BAPA and two with L-BAPA. There were also three control samples (not shown).

The run consisted of 7 samples; two with L-BAPA, two with DL-BAPA and three different control samples. The run was performed once with Protanal and once with Kelton.

4.2.2 Biocatalysis in Purified Manugel DMB

Manugel DMB was purified by dialysis. A solution of 5 % (w/v) Manugel DMB was prepared. Dialysis was performed for 5 days with deionized water that was changed once a day. The membrane had a cut off of 12-14000 Daltons. After dialysis the solution was freeze dried. Biocatalysis was then performed with samples of the purified manugel. Two samples with DL-BAPA were made and one sample with L-BAPA.

4.2.3 Biocatalysis with L-ZAPA and Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide in Manugel DMB

All samples were prepared according to section 4.1.1 except for the substrate solutions that were different. The run consisted of 7 samples. Two samples contained L-ZAPA, one sample L-ZAPA and D-BAPA, two samples Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide and two samples Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide and D-BAPA. The final concentrations in the samples were of polymer 1 % (w/v) and of enzyme 127 nM. The concentration of substrate was 133 µM and concentration of D-BAPA (in the samples that contained D-BAPA) was 133 µM.

The substrate L-ZAPA was solubilised in 32.5 % DMSO and L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide in 25 % acetonitrile. No control samples were made.

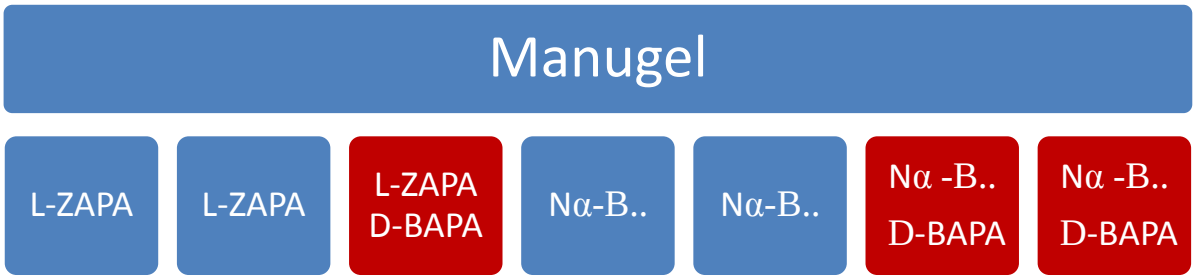

Figure 14. Schematic illustration of the different samples in the experiment. Two samples contained L-ZAPA, one sample L-ZAPA and D-BAPA, two samples Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide and two samples Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide and D-BAPA. The experiment was made once.

Handling of Absorbance Data

The reaction with Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide as substrate produced an instantaneous yellow color after adding enzyme and mixing. Because of this the initial absorbance of the samples were not corrected to zero.

4.3 Determination of Kinetic Constants

Biocatalysis was conducted at different concentrations of substrate in both buffer and Manugel DMB at 37°C. Every run consisted of seven samples with increasing concentration of L-BAPA. All samples were prepared as previously (see 4.1.1) except for different concentrations of L-BAPA solution.

The final concentration of L-BAPA in the alginate samples were as follows: 67, 133, 200, 267, 400, 533 and 1067 µM. All L-BAPNA solutions were made with 20 % DMSO. The experiment was repeated three times with the same concentrations.

The buffer samples were made using many different concentrations of L-BAPA solution in three different runs. The final concentration of L-BAPA in the samples were in run 1; 67, 133, 200, 267, 400, 533 and 1067 µM, in run 2; 67, 267, 533, 800, 1067, 1333 µM and in run 3; 67, 200, 267, 400, 533, 800, 1067 µM.

4.4 Analysis of Trypsin Conformation by Fluorescence Spectroscopy

Fluorescene spectra was recorded on a RF-5000 recording spectrofluorophotometer. The excitation wavelenght was set to 280 nm. Solutions of alginate and enzyme was mixed directly in the cuvette. Concentrations of the enzyme used in theese experiments were 127 nM and 1 µM.

5. Results

In this section the results of the experiments are presented.

5.1 Catalysis in Different Polymer Solutions and Buffer

In this section the results from the kinetic measurements in the different polymers are shown. For results of the control sample runs the reader is refereed to appendix. The scale in the figures is the same except for Figure 16 and Figure 20.

5.1.1 Buffer

Below the results from catalysis with trypsin in buffer is shown. Product concentration (µM) is given as a function of time (hours).

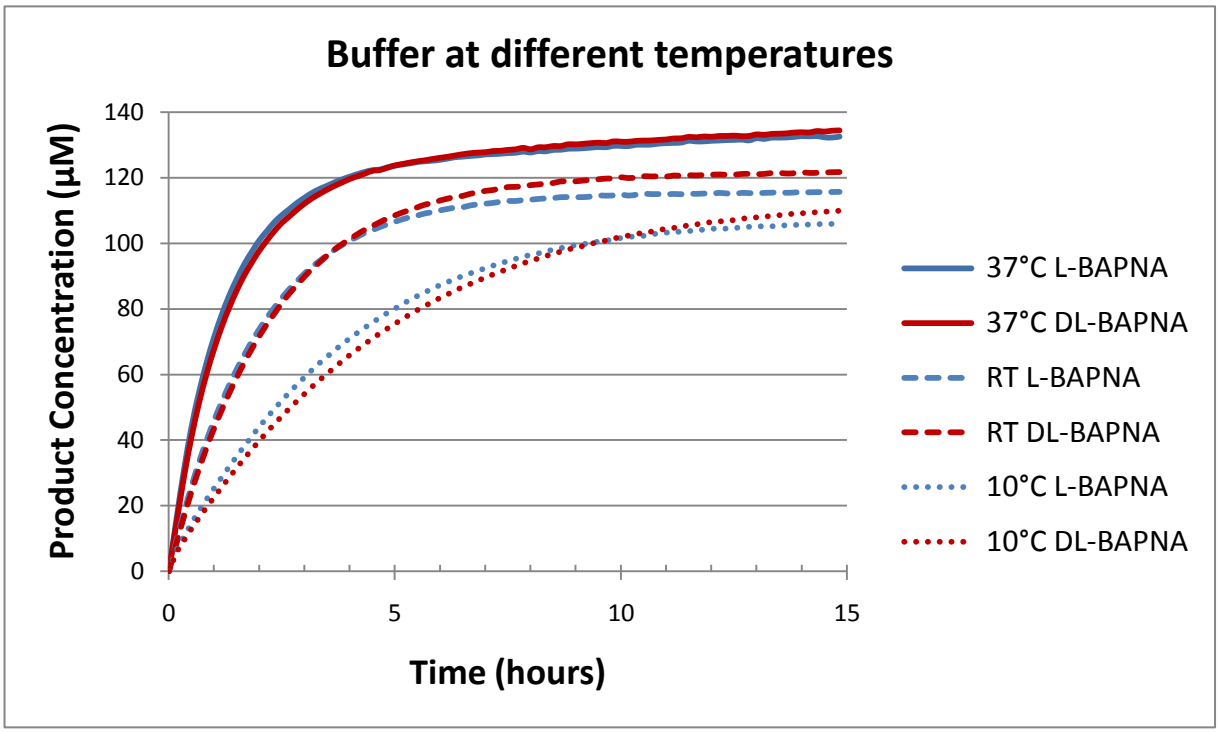

Figure 15. Biocatalysis with trypsin in buffer at different temperatures. Product concentration (µM) as a function of time (hours).

No significant difference between experiments with L-BAPA and DL-BAPA can bee observed in the figure. The initial activity of the enzyme (represented by the first hour on the figure) is increasing with the temperature, which is in agreement with what is known about trypsin kinetics.

Control samples showed little variation (<1 µmol) of product and thus no compensation was made in the representation of the results.

5.1.2 Alginate Manugel DMB

Below the results from catalysis with trypsin in the alginate product Manugel DMB is shown. Product concentration (µM) is given as a function of time (hours).

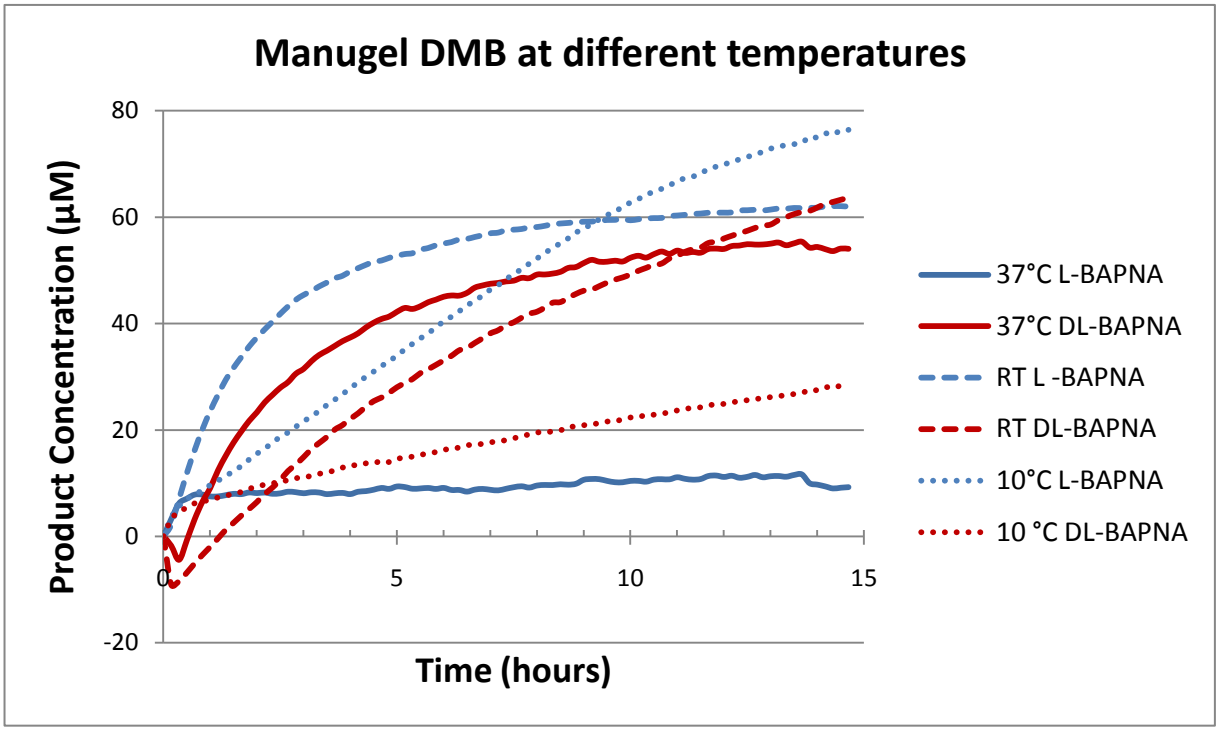

Figure 16. Biocatalysis with trypsin in Manugel DMB at different temperatures. Product concentration (µM) as a function of time (hours). Note that the scale is different from the other result figures.

This figure shows significant differences between experiments made at different temperatures. At 10°C addition of D-BAPA in the system induces a strong inhibition of the reaction. This inhibition is lower for the experiments at room temperature but still pronounced. At 37°C however there is almost no reaction with only L-BAPA whereas with DL-BAPA the rate of the biotransformation is much higher.

All control samples showed significant variation over time (see Appendix). The control samples absorbance was deducted from the corresponding samples.

5.1.3 Polyethyleneimine

Below the results from catalysis with trypsin in polyethyleneimine is shown. Product concentration (µM) is given as a function of time (hours).

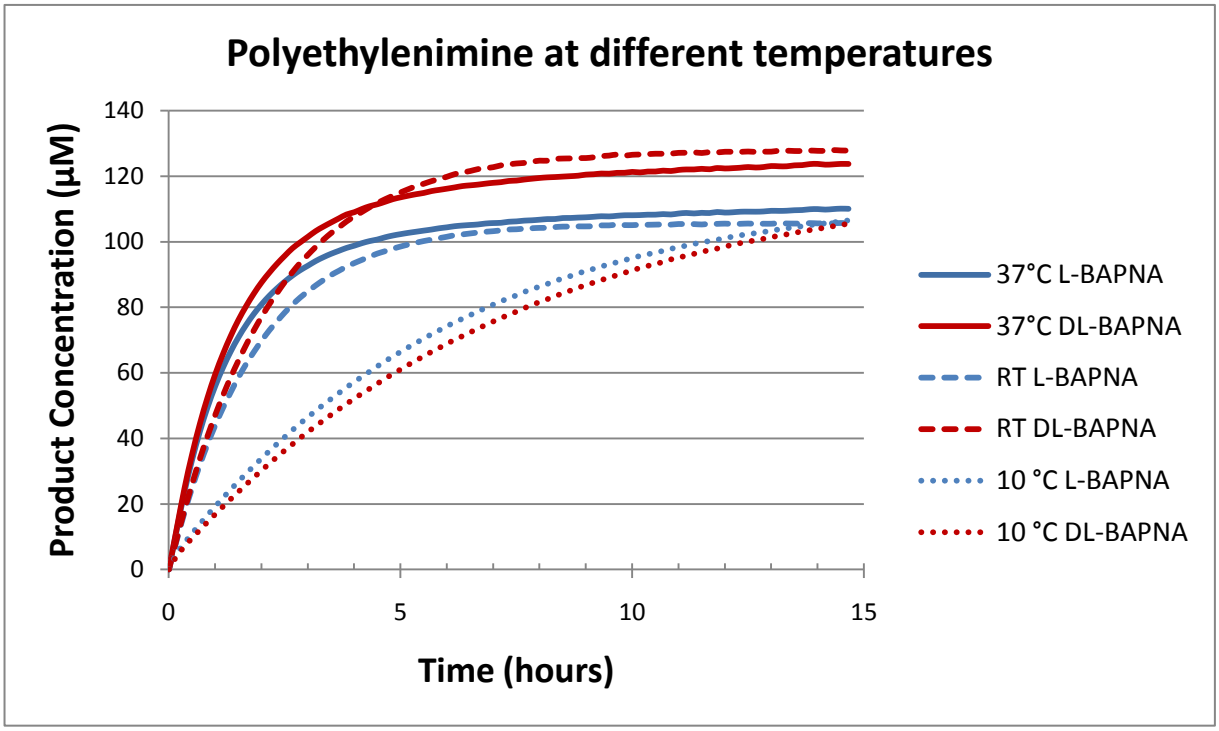

Figure 17. Biocatalysis with trypsin in Polyethyleneimine at different temperatures. Product concentration (µM) as a function of time (hours).

For this experiment, the addition of D-BAPA in the system induces a small activation of the reaction at room temperature and 37°C and almost no difference is seen at 10°C.

Control samples for 10°C showed negligible variation (<1 µmol) of product concentration and thus no compensation was made in the representation of the results. However the control samples at 37°C and room temperature showed some variation (see Appendix), and the control sample values of absorbance was deducted from the corresponding samples.

5.1.4 Polyethylene glycol

Below the results from catalysis with trypsin in polyethylene glycol is shown. Product concentration (µM) is given as a function of time (hours).

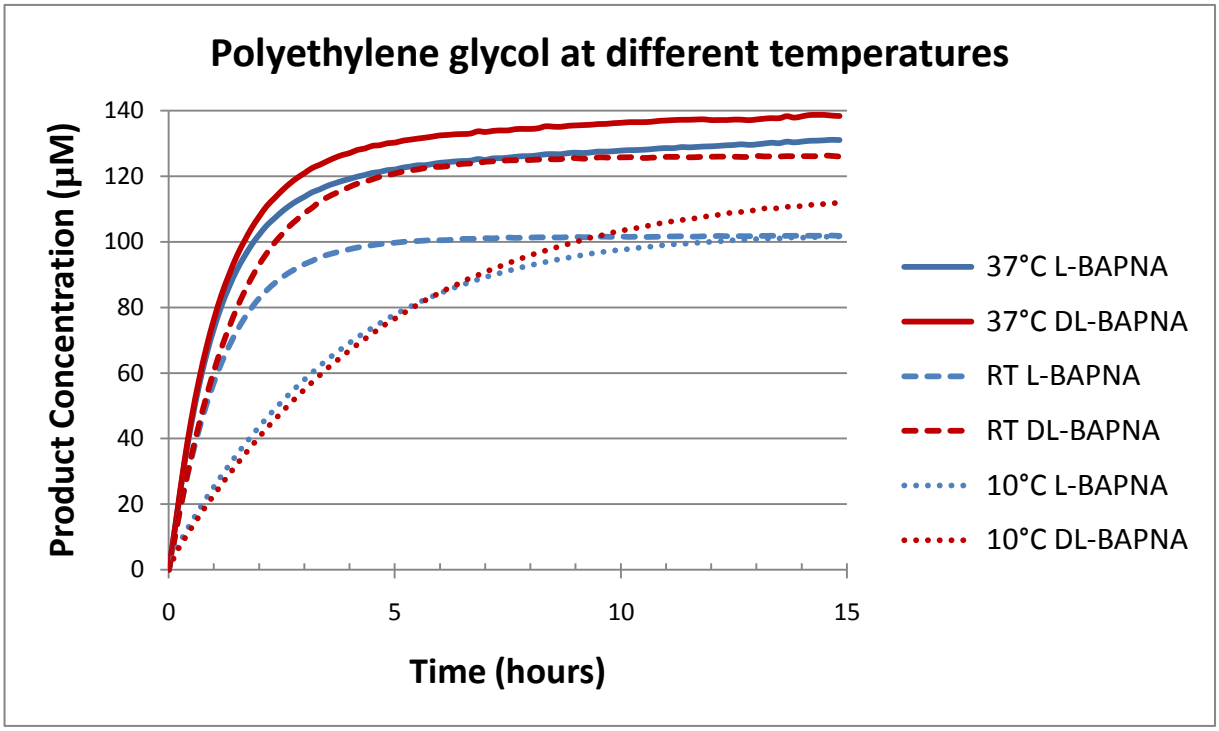

Figure 18. Biocatalysis with trypsin in Polyethylene glycol at different temperatures. Product concentration (µM) as a function of time (hours).

Final product absorbance for 37°C DL-BAPA is a bit higher than possible, full conversion to product would correspond to 133 µM. This indicates some slight measurement error. The results are very similar to what is seen in PEI.

Control samples for 10°C and room temperature showed negligible variation (<1 µmol) of product concentration and thus no compensation was made in the representation of the results. However the control samples at 37°C showed some variation (see Appendix), and the control sample values of absorbance was deducted from the corresponding samples.

5.1.5 Polyacrylic acid

No reaction occurred at room temperature in polyacrylic acid during the 15 hours of the experiment. This is in agreement with literature (see background 2.3.4). No experiments were made at 10°C or 37°C.

5.1.6 Polyvinylsulfuric acid

Below the results from catalysis with trypsin in polyvinylsulfuric acid is shown. Product concentration (µM) is given as a function of time (hours).

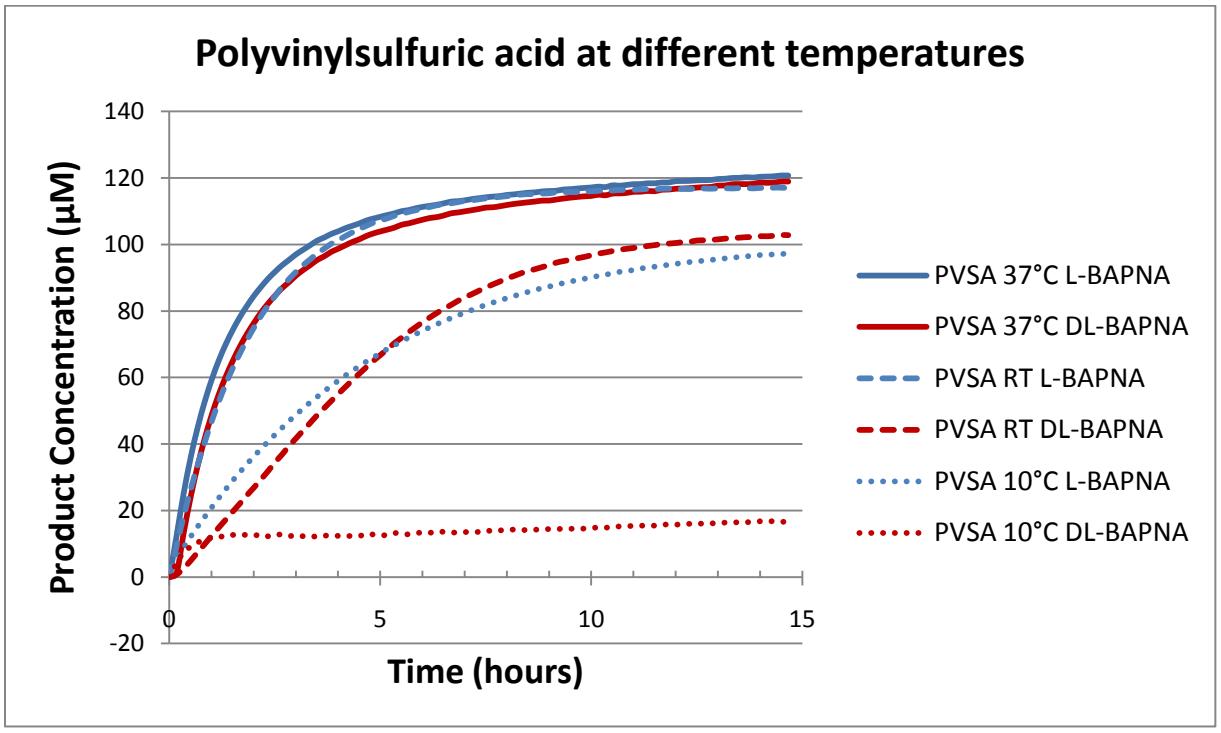

Figure 19. Biocatalysis with trypsin in polyvinylsulfuric acid at different temperatures. Product concentration (µM) as a function of time (hours). The low reaction with DL-BAPA at 10°C can be attributed to observed precipitate formation and can thus be considered as trivial.

The low reaction with DL-BAPA at 10°C can be attributed to observed precipitate formation and can be considered as trivial. At 37°C, no significant difference is seen between the two experiments whereas at room temperature, addition of D-BAPA induces a moderate inhibition of the reaction.

Control samples showed little variation (<1 µmol) of product and thus no compensation was made in the representation of the results.

5.1.7 Xanthan

Below the results from catalysis with trypsin in xanthan is shown. Product concentration (µM) is given as a function of time (hours).

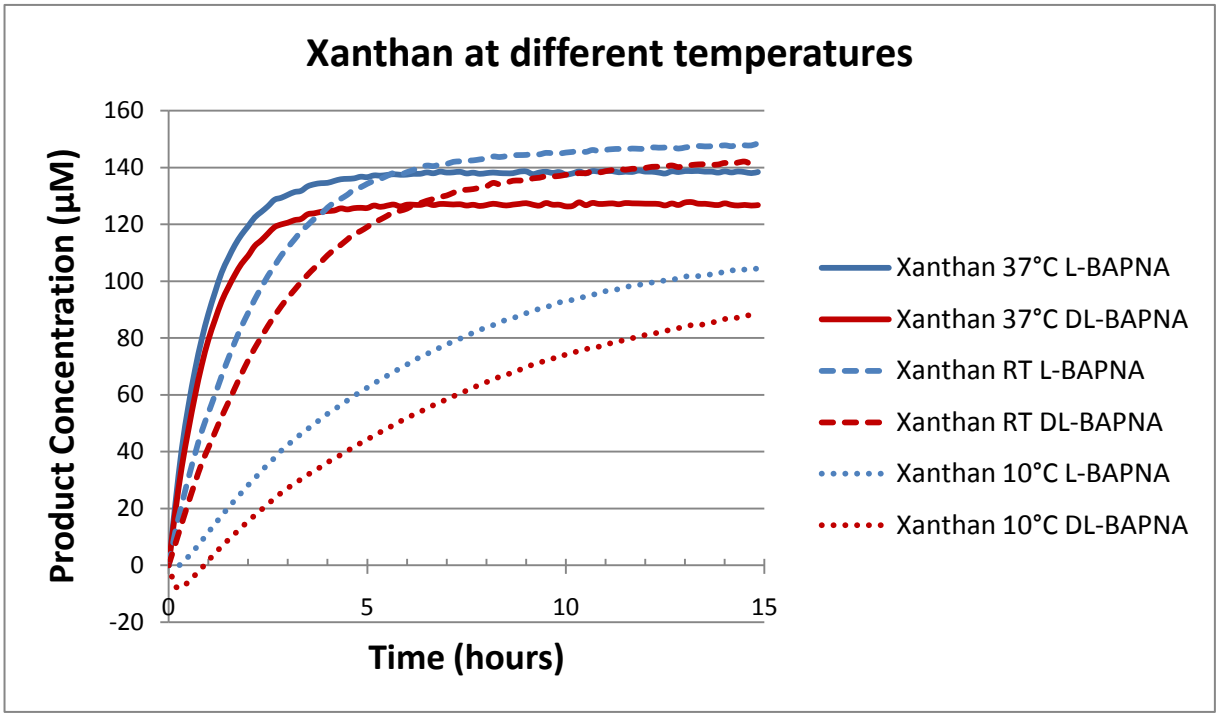

Figure 20. Biocatalysis with trypsin in Xanthan at different temperatures. Product concentration (µM) as a function of time (hours). Note that the scale is slightly different from the other figures.

Control samples showed significant absorbance values (see appendix) and have been subtracted from the results given. Final product absorbance is a bit higher than possible, full conversion to product would correspond to 133 µM. This indicates some measurement error, possibly because of the highly viscous solution being prone to bubble formation upon mixing.

Nevertheless, at each temperature, this figure shows that working with DL-BAPA induces a small inhibition of the reaction compared to the reaction with only L-BAPA.

5.1.8 Carboxymethylcellulose

Below the results from catalysis with trypsin in carboxymethylcellulose is shown. Product concentration (µM) is given as a function of time (hours).

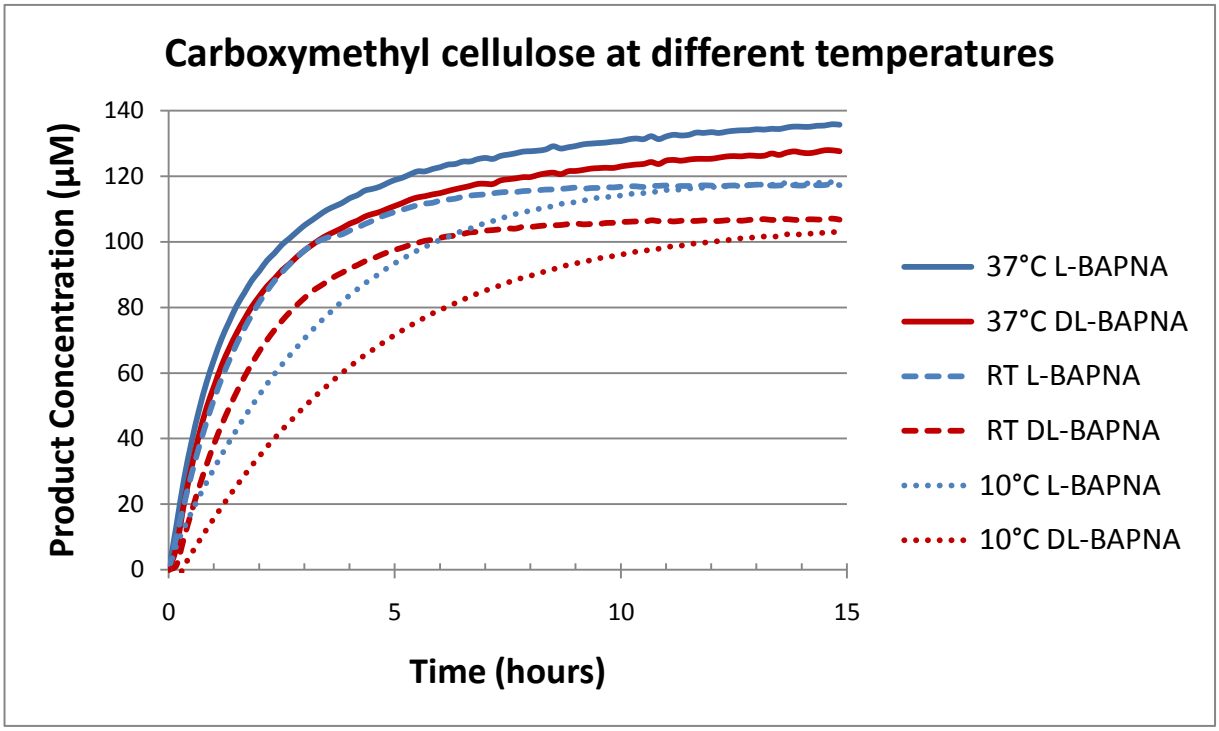

Figure 21. Biocatalysis with trypsin in Carboxymethyl cellulose at different temperatures. Product concentration (µM) as a function of time (hours).

Control samples showed little variation (<1 µmol) of product and thus no compensation was made in the representation of the results.

As for experiments with xanthan, with carboxymethyl cellulose DL-BAPA induces an inhibition of the reaction to some extent at all the tested temperatures.

5.2 Determination of Kinetic Constants

Below the results from the experiments in section 4.3 is given. Substrate concentration was varied and the initial conversion rate of substrate to product was calculated for the different substrate concentrations. In Figure 22 a) the rate in buffer at 37°C is plotted as a function of substrate concentration. In Figure 23 a) the rate in Manugel DMB at 37°C is plotted as a function of substrate concentration. In Figure 22 b) and Figure 23 b) the reciprocal of the rate of the reaction is given as a function of the reciprocal of the substrate concentration.

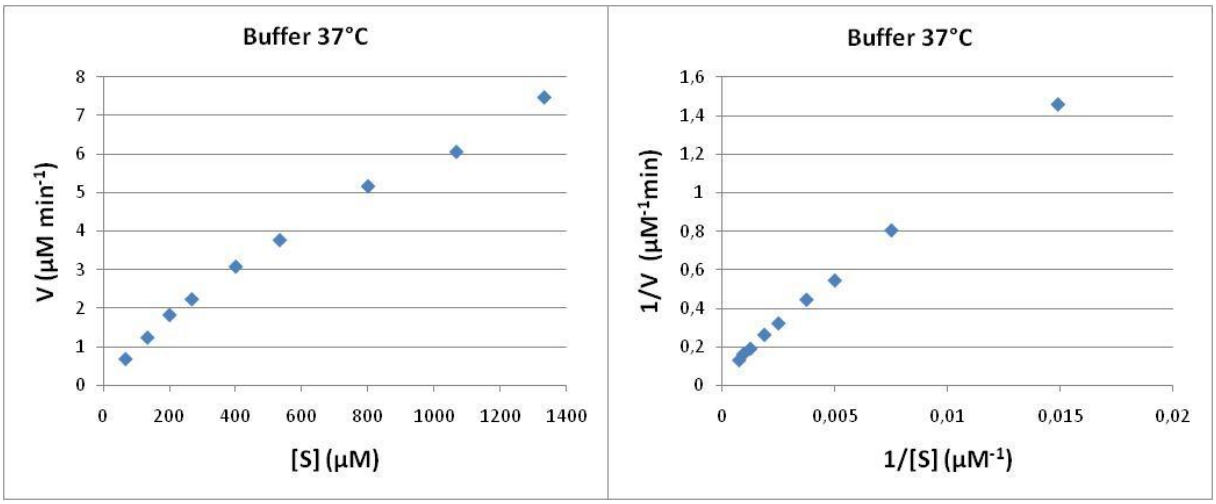

Figure 22. a) The rate of substrate conversion as a function of substrate concentration in buffer. b) Enzyme concentration was 127 nM.

Kinetic constants were calculated for the buffer experiment to; K_m=1.1 mM and V_{max}=11.8 µmol min^{-1}

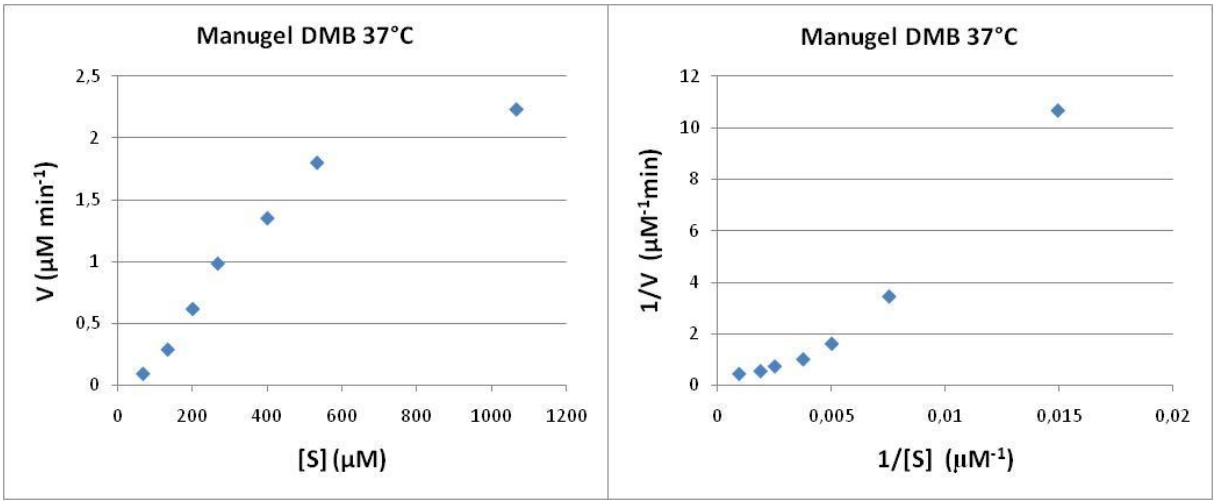

Figure 23. a) The rate of substrate conversion as a function of substrate concentration in Manugel DMB. b) Enzyme concentration was 127 nM.

As non Michaelis-Menten kinetics is observed (see Figure 23) in Manugel DMB no determination of kinetic constants could be made.

5.3 Catalysis in Solutions of Different Alginate Products

As the kinetics in Manugel was significantly different from the other polymers the natural continuation was to examine other alginate products. The results from these experiments are given in this section.

5.3.1 Catalysis in Solutions of Different Alginate Products

Below a comparison of the biotransformation process in different alginate products is given.

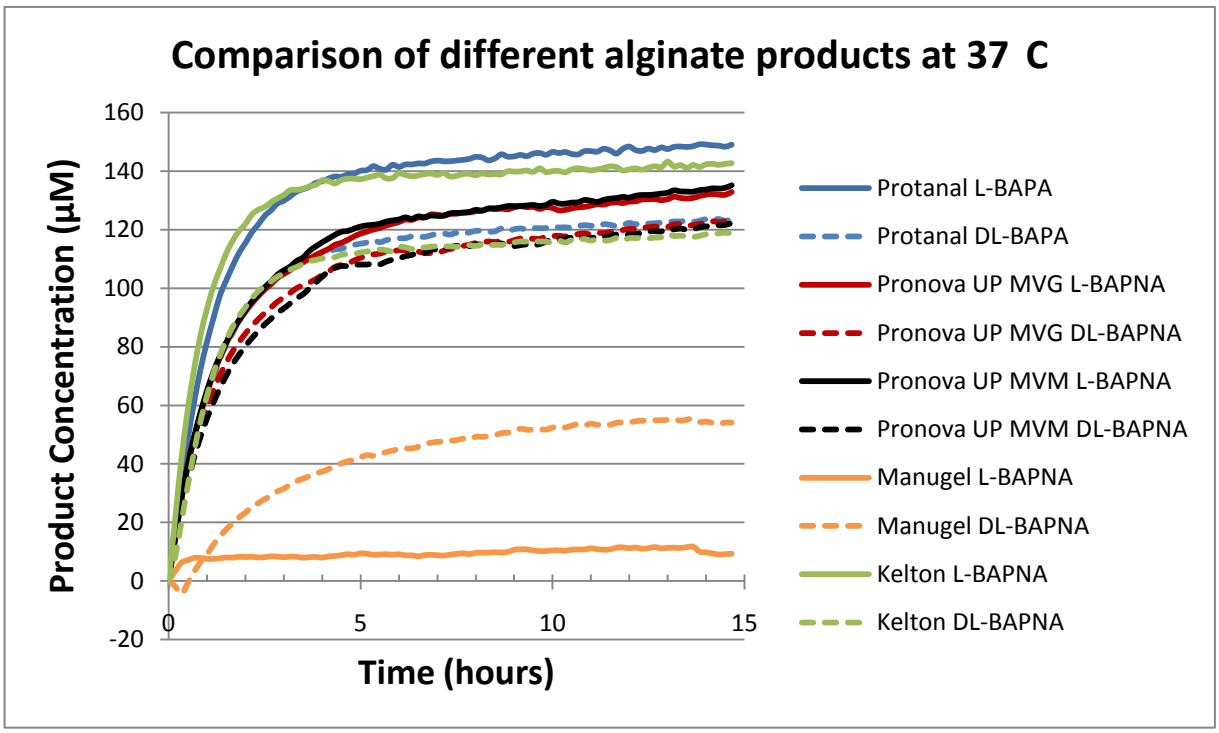

Figure 24. Comparison of biocatalysis in different alginate products. The results in Manugel DMB is significantly different from the results in other alginate products.

The results show that the biocatalysis in Manugel DMB significantly differs from the other alginate products. All alginate products tested except for Manugel all show inhibition of the reaction by D-BAPA. Final product absorbance for Kelton is a bit higher than possible, full conversion to product would correspond to 133 µM. This indicates some slight measurement error.

5.3.2 Biocatalysis in Purified Manugel DMB

Below the results from biocatalysis in purified Manugel DMB are compared to previous results with Manugel DMB.

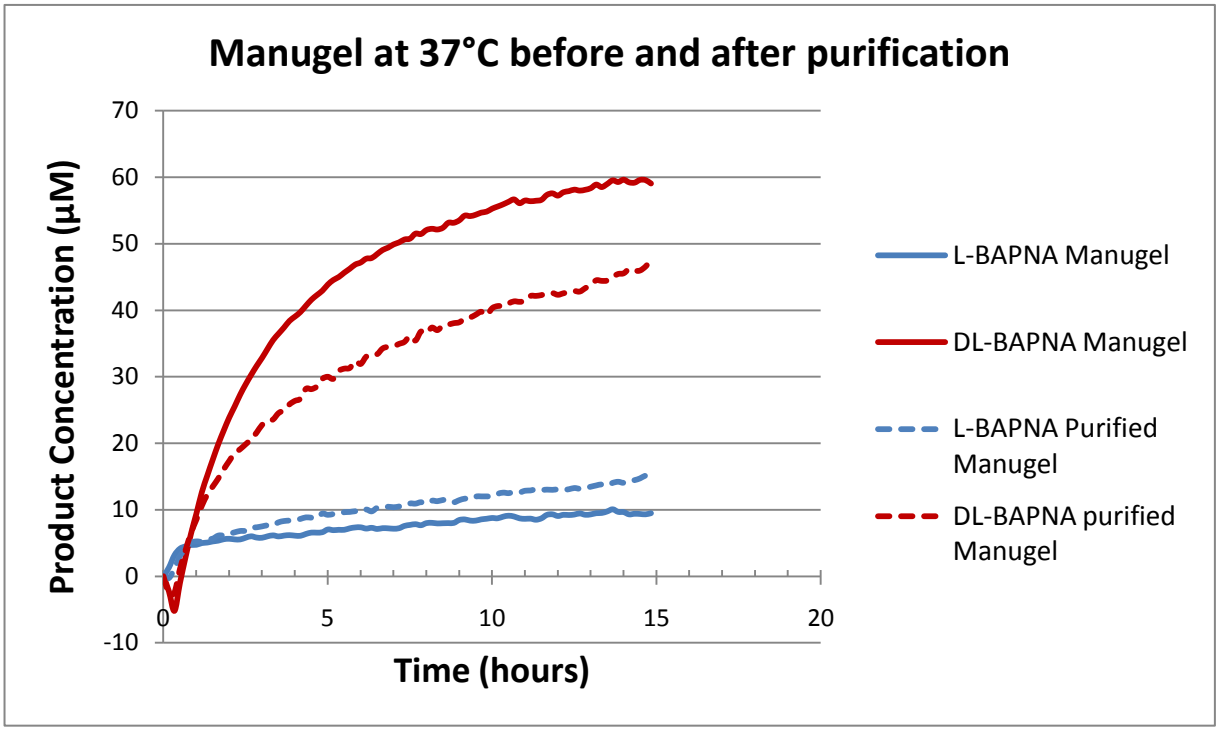

Figure 25. Comparison of biocatalysis in Manugel DMB before and after purification by dialysis. No correction by control samples were made in this graph.

The results indicate that there is no significant difference between the purified and non purified Manugel DMB.

5.3.3 Biocatalysis with L-ZAPA and Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide in Manugel DMB

In this experiment two other substrates were used; the substrate Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide which is very different to L-BAPA and the substrate L-ZAPA which is highly similar to L-BAPA. Further the effect of the inhibitor D-BAPA was also tested. The results are shown in Figure 26. Biocatalysis with L-ZAPA and Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide in Manugel DMB at 37°C.

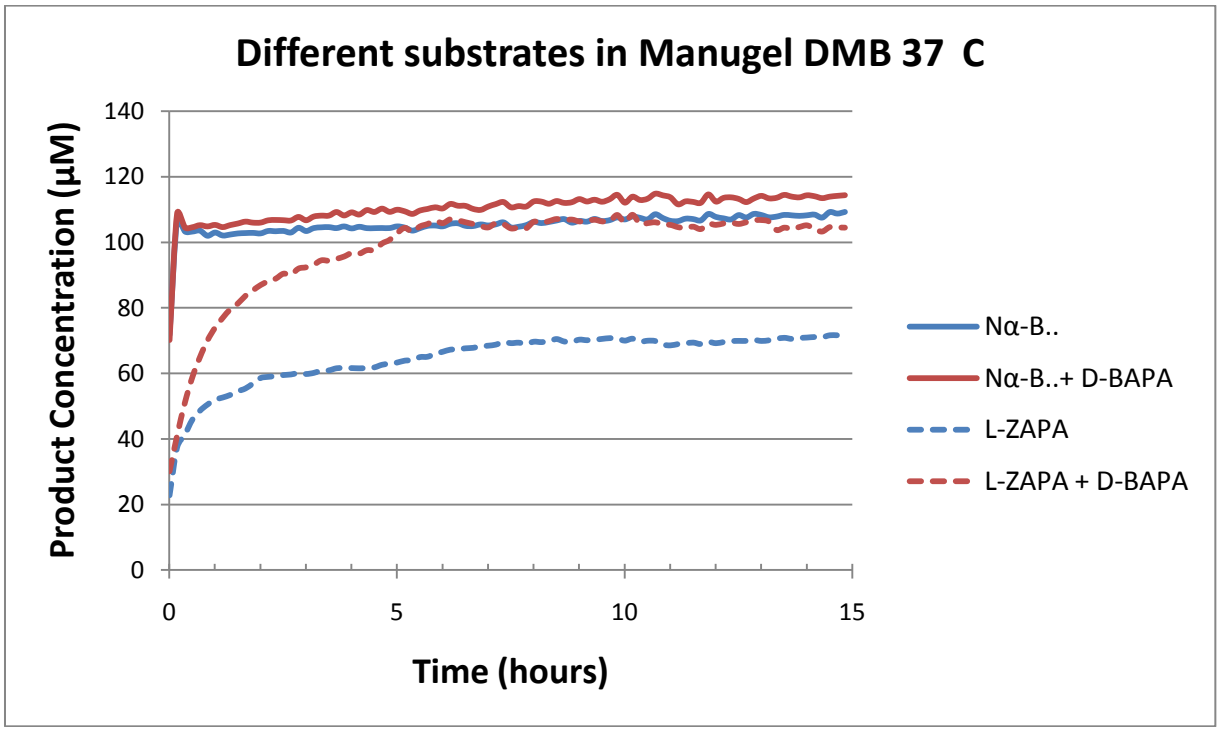

Figure 26. Biocatalysis with L-ZAPA and Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide in Manugel DMB at 37°C.

No modifications were made to the data in the graph. As the reaction with Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide proceeded to rapidly to detect the start no initial absorbance was deducted from the samples. However previous experiments with L-BAPA and DL-BAPA suggest that an initial absorbance corresponding to 20 µM product can be approximated giving a final absorbance of about 90 µM which is low.

As with experiments with BAPA, addition of D-BAPA to experiment with L-ZAPA induces an increase of the biotransformation rate compared to the experiment without D-BAPA.

5.4 Analysis of Trypsin Conformation in Alginate Solution and Buffer by Fluorescence Spectrometry

In buffer the initial fluorescence without enzyme was negligible. However in the ultrapure alginates some fluorescence was still present. This makes measurements at the low enzyme concentration of 127 nM unreliable. Measurements at a higher enzyme concentration of 1 µM indicated no difference in conformation between buffer, MVM or MVG alginates.

The fluorescence of Manugel DMB was too strong to make any measurements.

6. Discussion

From the results in section 5.1 it is strongly indicated that the D-BAPA activation observed in Manugel DMB is not present in other polymers. Additionally experiments in CMC and Xanthan suggest that the effect is not strongly related to polysaccharides or even the carboxylic group.

The inhibition of trypsin by polyacrylic acid (which also contains a carboxylic group) is previously known (see background). The reason for the inhibition is believed to partly be caused by formation of an ion-complex between the polymer and the substrate used N-a-benzoyl-L-arginine-ethylester. The strong inhibition in the experiment conducted in this study is likely due to the low substrate concentration. Further as there is no counter ion present in the PAA solution it is likely that the formation of a complex between the positively charged BAPA and negatively charged carboxylic group occurs. This raises some thoughts about the possibility of low substrate concentration as a possible explanation for the observed activation by D-BAPA in Manugel DMB. As the total BAPA concentration is higher in presence of inhibitor the result would be a larger portion of free substrate to bind to the enzyme.

In section 4.3 several experiments were conducted with increasing L-BAPA concentration. The results strongly indicate that the inhibition is stronger at low substrate concentrations. The substrate concentration at which the reaction rate increases substantially is in the range of 267-400 µM L-BAPA (see Figure 23). The plot in figure 23 b shows two regions, at a concentration of 400 µM and above the data is quite linear, similar to what is seen in buffer. The concentration of DL-BAPA in the previous experiments (see Figure 16) was 267 µM thus a concentration in the range before the linear region. The low reaction rate at low substrate concentrations could perhaps be due to a low concentration of BAPA. It could be that low BAPA concentrations form a complex with Manugel DMB and that the saturation point occurs slightly before 267 µM. However if such a complex would be formed it could not be interactions of every carboxylic group and every BAPA-molecule. The ratio of carboxylic groups to BAPA molecules at a concentration of 1 wt % alginate and 267 µM BAPA is 21:1. Because of the complex structure of alginate it is possible that only certain parts of the polymer can interact with the BAPA. Perhaps this alginate is particularly rich in alternating GM sequences compared to the other alginate products. Only the total M and G content of the alginate products are known and not the sequence of the residues. Also the large enzyme (23 000 Daltons) has a net positive charge and could also block some of the carboxylic groups by interactions.

Further in Figure 16 it is apparent that not all substrate is converted to product in Manugel DMB. The final product concentration with DL-BAPA is 60 µM which can be compared to the expected value of 133 µM. Biotransformation in all other polymers show a final product concentration in the range of 100-145 µM. As the product concentration should not exceed 133 µM it is definitely some experimental error present. This is likely due partly to the difficulty of weighing small amounts exactly and also partly due to the formation of bubbles when mixing. In the polymers with high viscosity (xanthan, alginate and cmc) bubble formation was particularly difficult to avoid. However it is clear that the final product formation in Manugel DMB is significantly lower than in all other solutions tested (except for PAA). This is strongly illustrated in Figure 24 where Manugel DMB is compared to other alginate products.

Further in Figure 24 it is shown that the D-BAPA activation is a Manugel DMB specific effect. A total of four other alginates were tested and they all showed quite similar kinetic curves. From Table 1 the specific properties of the different alginate products are summarized. When examining the table none of the specified properties for Manugel DMB are unique. Pronova UP MVG is highly similar to Manugel DMB in the aspect of mannuronic and guluronic acid content (M/G ratio) and the molecular weight is in a similar range. One cannot entirely exclude that it could be a combined effect of M/G

ratio and a specific molecular weight range that causes the effect. However it seems unlikely as the molecular weights are only approximate averages.

Two of the other alginates tested were also of food grade perhaps suggesting that the degree of purification of the Alginate is not the cause. However as the effect was not present in any other alginate tested, further purification of Manugel DMB was conducted by dialysis. In Figure 25 it can be seen that the purification of Manugel DMB by dialysis had no significant effect on the biotransformation kinetics.

That the activation of the reaction by D-BAPA in Manugel is atleast partly due to the BAPA is supported by the experiment with the zwitterionic substrate. As can be seen in Figure 26 the reaction with the zwitterionic substrate Nα-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide takes place with a high rate constant and the final absorbance reaches higher values than with L-BAPA and DL-BAPA. What is interesting is that no significant difference in reaction can be seen with D-BAPA compared to without D-BAPA for the zwitterionic substrate. A similar effect to that seen with L-BAPA is observed for the substrate L-ZAPA which is very similar in structure to L-BAPA. This indicates that at least part of the effect is related to the BAPA and molecules similar to it. The final absorbance however is somewhat lower than could be expected; it can be approximated to about 90 μM which is lower than the expected value of 133 μM. However without buffer as a reference it is impossible to say if this substrate is converted into product to the same extent as L-BAPA.

However an important point that cannot be disregarded is that the final absorbance is the same for L-ZAPA with D-BAPA as it is for the zwitterionic substrate (see Figure 26). This is difficult to explain with a theory of BAPA forming a complex with the polymer. It is likely that such a complex would gradually move towards a solely D-BAPA-Manugel DMB complex as L-BAPA is depleted through reaction, but it is unlikely that both final product amounts should coincide. Also 90 μM is low for final product formation compared to results with L-BAPA in other polymers and buffer.

One theory that has been suggested is that the effect could be due to a conformational change of the enzyme in interaction with the alginate. Due to quenching of the tryptophan fluorescence by the fluorescence of Manugel DMB the conformation of the enzyme could not be studied. The theory cannot be disregarded but the experiment with the zwitterionic substrate in Manugel DMB does raise some question marks regarding D-BAPA as an enabler of the reaction. Further no conformational change could be observed in Pronova UP MVM or Pronova UP MVG.

7. Conclusion

The activation by D-BAPA is a highly specific effect present only in Manugel DMB out of the 5 alginates that were tested. Nor could a similar effect be observed in any of the other polymer solutions tested. The effect was not present when a change of the substrate to a zwitterionic substrate was made indicating that perhaps the effect is related to BAPA interactions with Manugel DMB alginate. As shown when varying the substrate concentration the effect is most pronounced at low substrate concentrations. Further the total amount of product formed in Manugel is significantly lower than in all other polymers, which may suggest that some substrate is not free to enter the enzyme.

8. Suggestions for Future Experiments

In order to fully investigate if there is a conformational change of the enzyme further purification of Manugel DMB should be conducted. Perhaps an organic solvent could be used during purification. If the solution's initial fluorescence could be significantly decreased it might be possible to study the fluorescence of trypthophan in Manugel DMB.

Further if no conformational change would be observed the solution could be titrated by D-BAPA. The shift of the fluorescence peak as the D-BAPA concentration increased could then be compared to the shift of the peak in buffer. If in fact the D-BAPA forms a complex with Manugel DMB the shift would not occur until after the polymer reached saturation.

It would also be useful to test other substrates, preferably a smaller substrate without the anionic group. Further it would be useful to repeat the experiment with the zwitterionic substrate in buffer.

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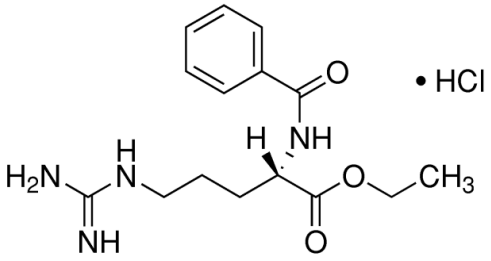
Appendix

The structure of N-α-benzoyl-L-arginine-ethylester



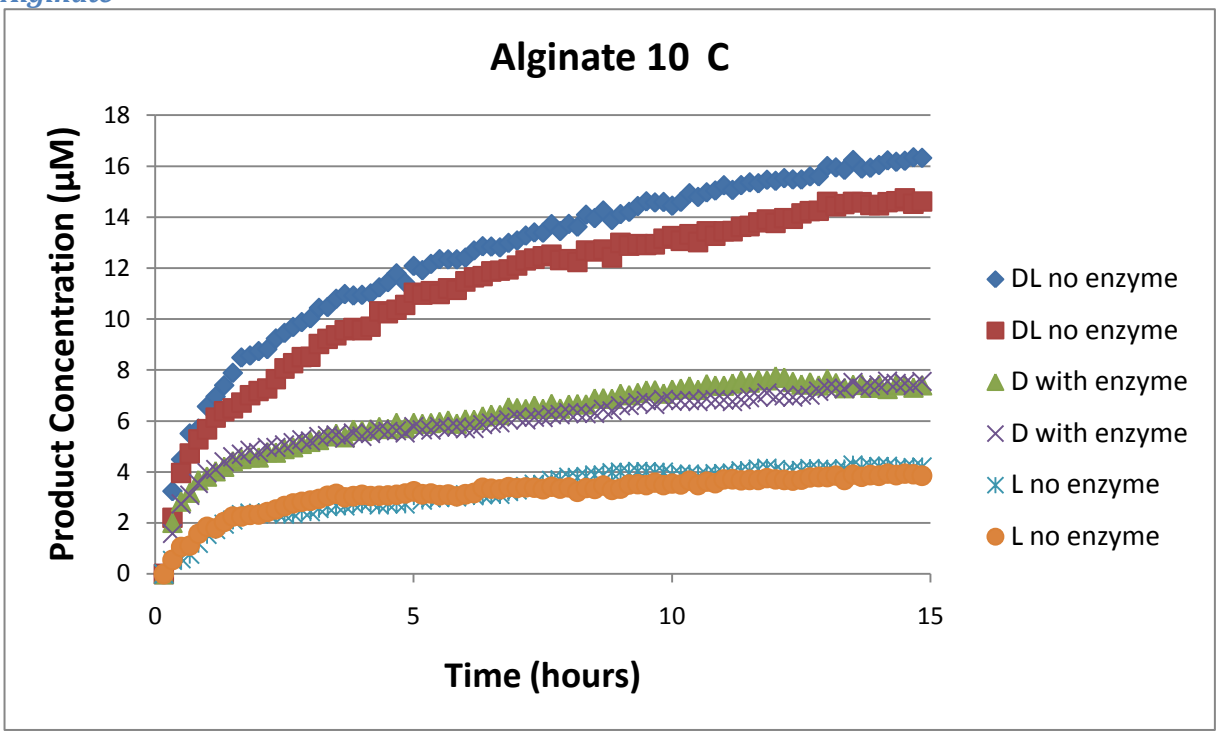
Results from control runs

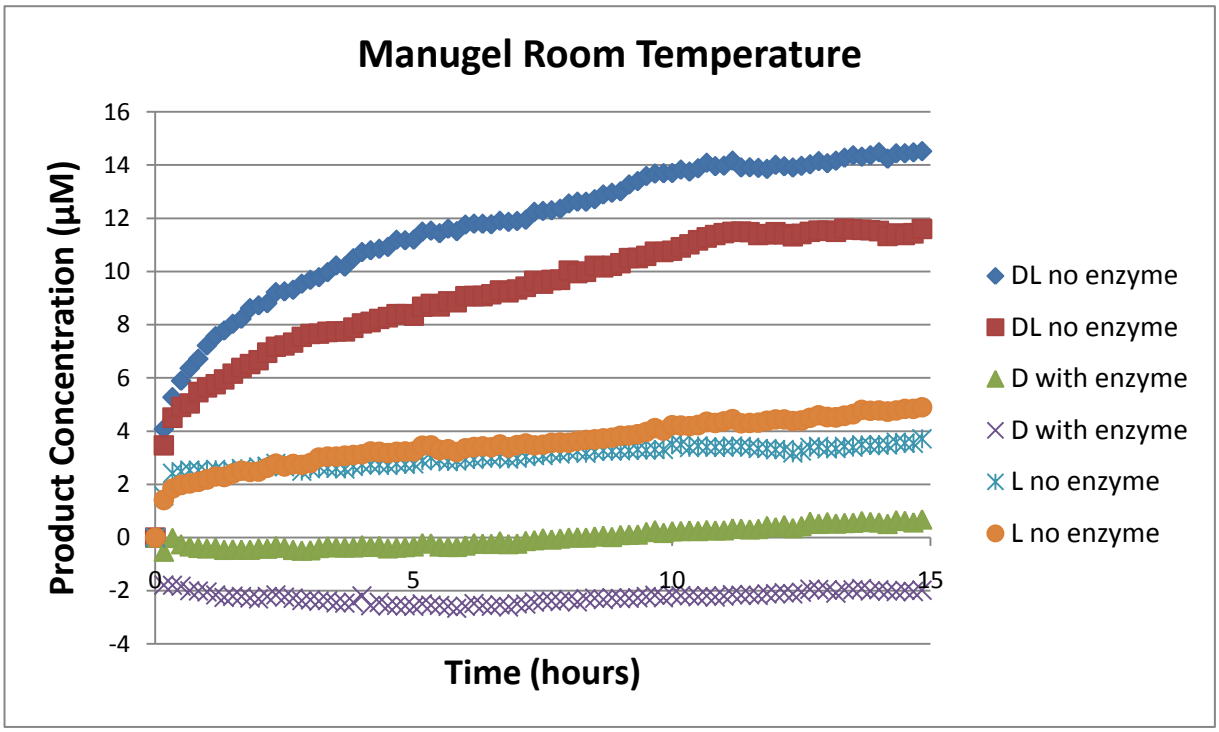
Below the results from the control runs are shown. Results that showed little or no change in absorbance has been omitted.

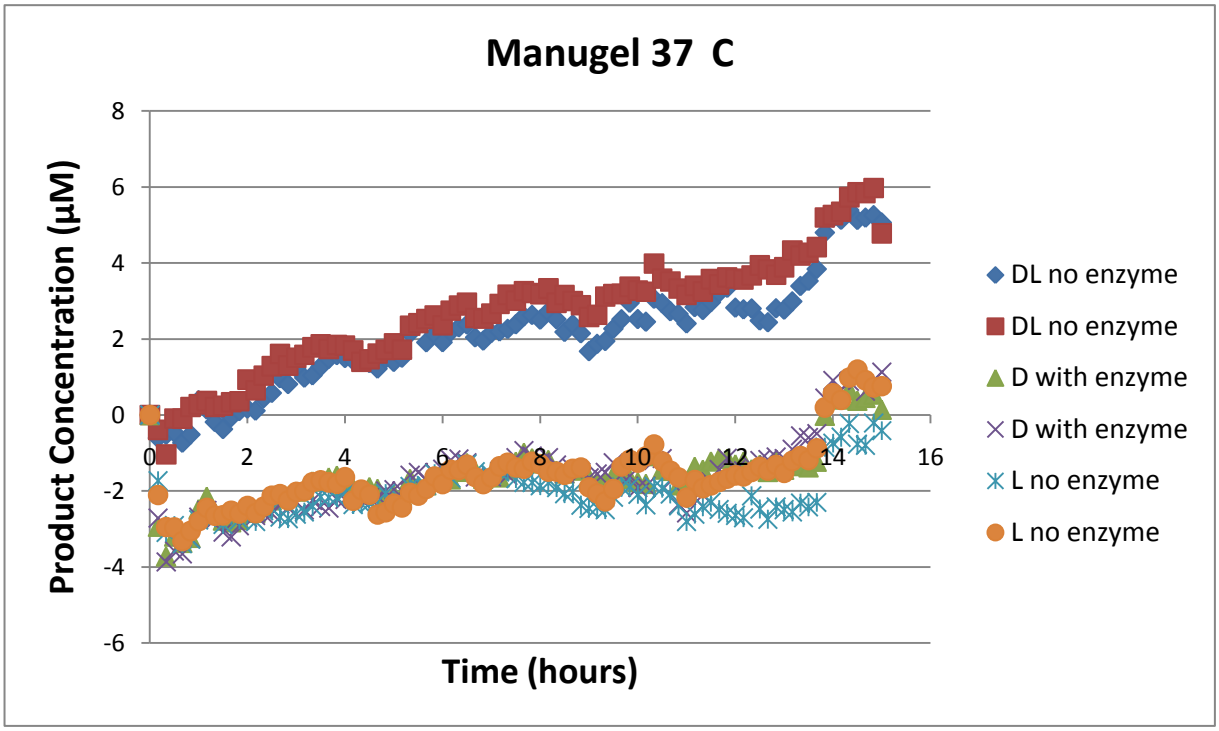
Buffer

All buffer control samples showed variation corresponding to less than 1 µmol of product and is thus not shown.

Alginate

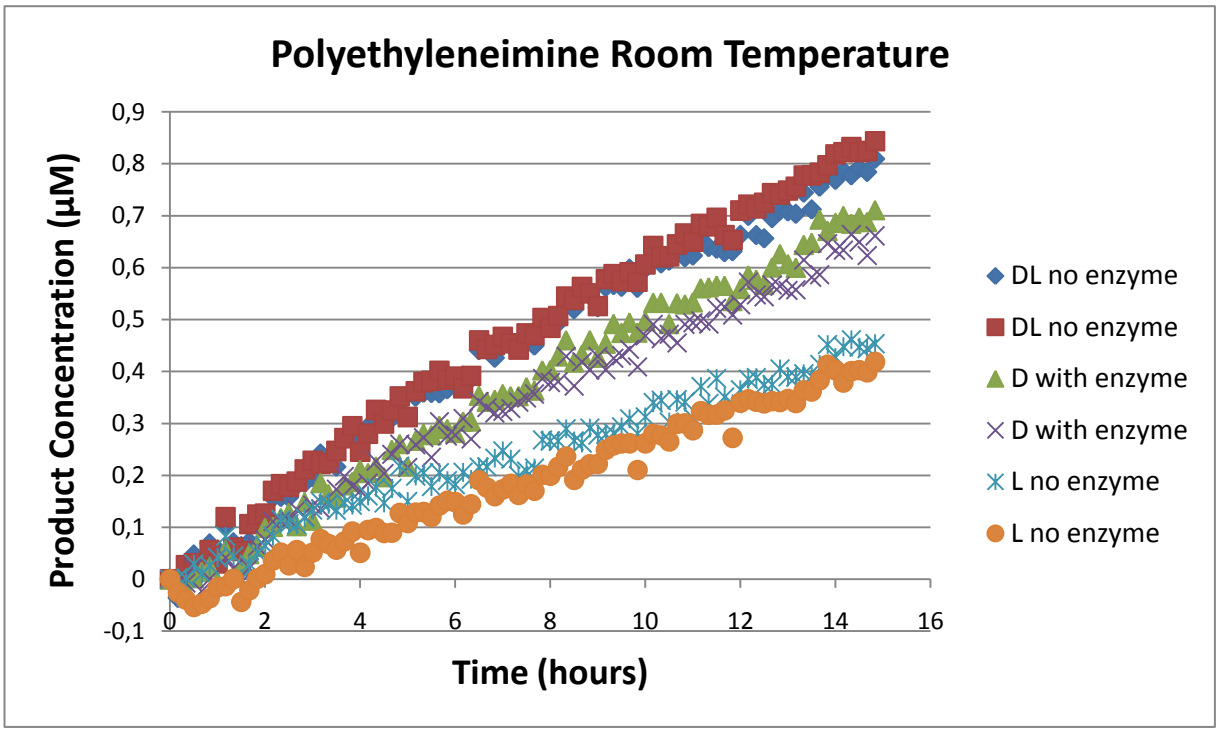


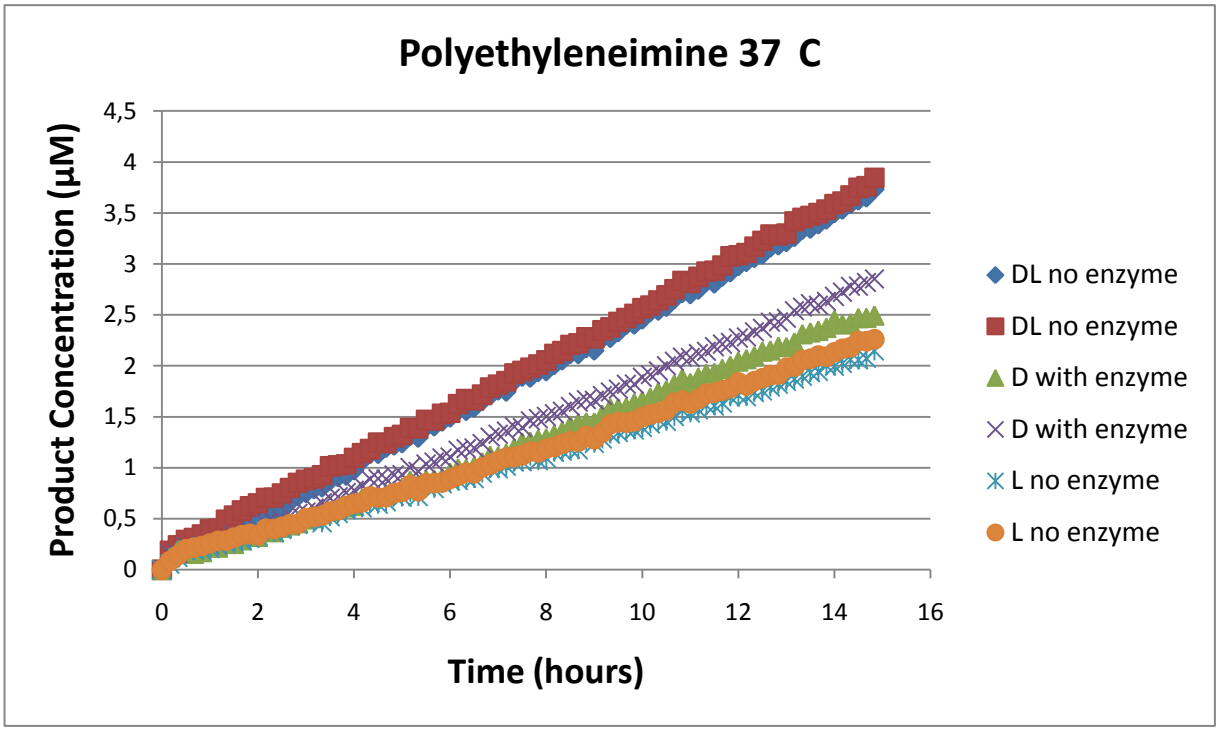




Polyethyleneimine

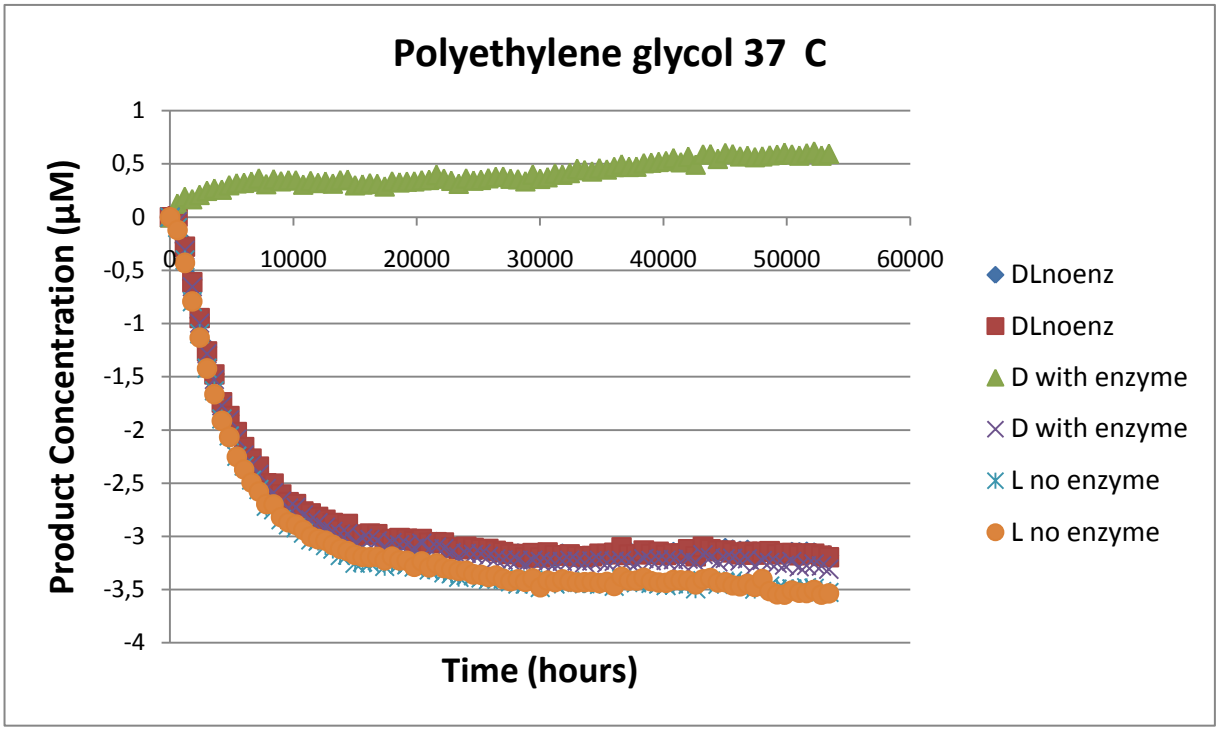
The control runs for PEI at 10°C showed negligeable variation in absorbance during 15 hours. Therefore only the experiments at the other temperatures are shown.


Polyethyleneimine Room Temperature
Product Concentration (µM)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
-0,1
0
2
4
6
8
10
12
14
16
Time (hours)
DL no enzyme
DL no enzyme
D with enzyme
D with enzyme
L no enzyme
L no enzyme


Polyethyleneimine 37 C
Product Concentration (µM)
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
0
2
4
6
8
10
12
14
16
Time (hours)
DL no enzyme
DL no enzyme
D with enzyme
D with enzyme
L no enzyme
L no enzyme

Polyethylene glycol

The control runs for PEG at Room Temperature and 10°C showed negligeable variation in absorbance during 15 hours. Therefore only the control run for 37°C is shown.



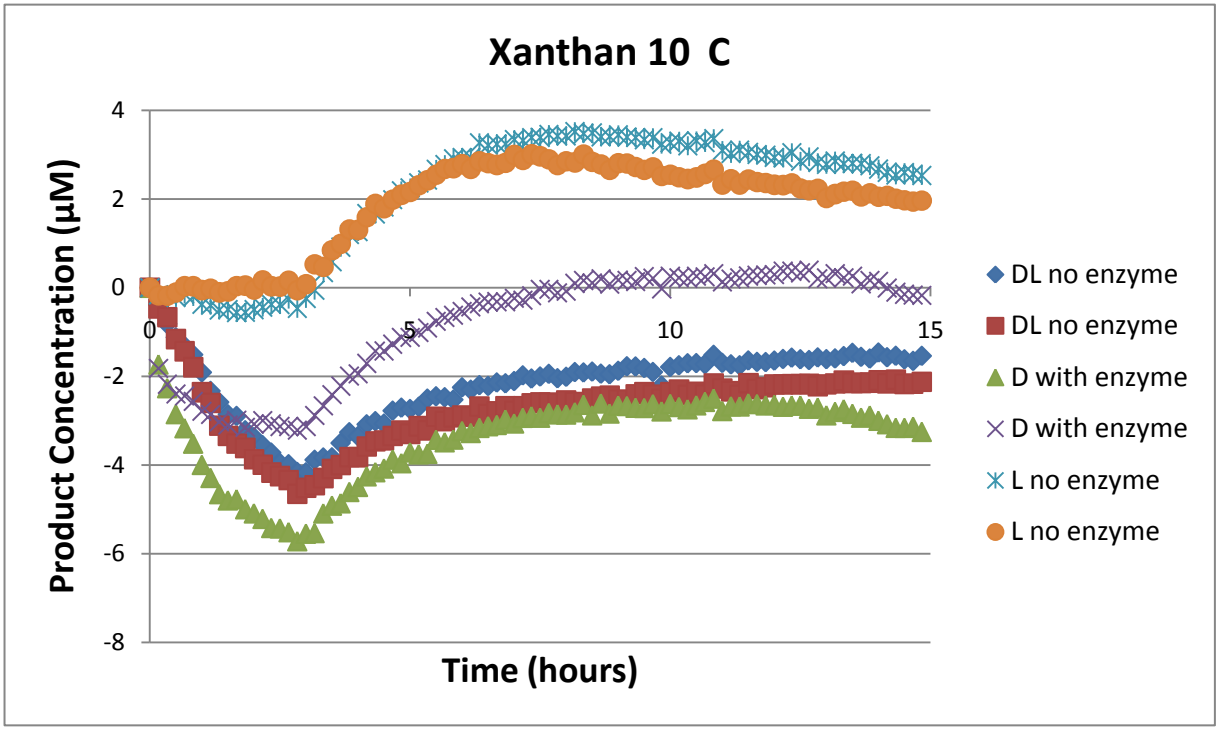
Polyacrylic acid

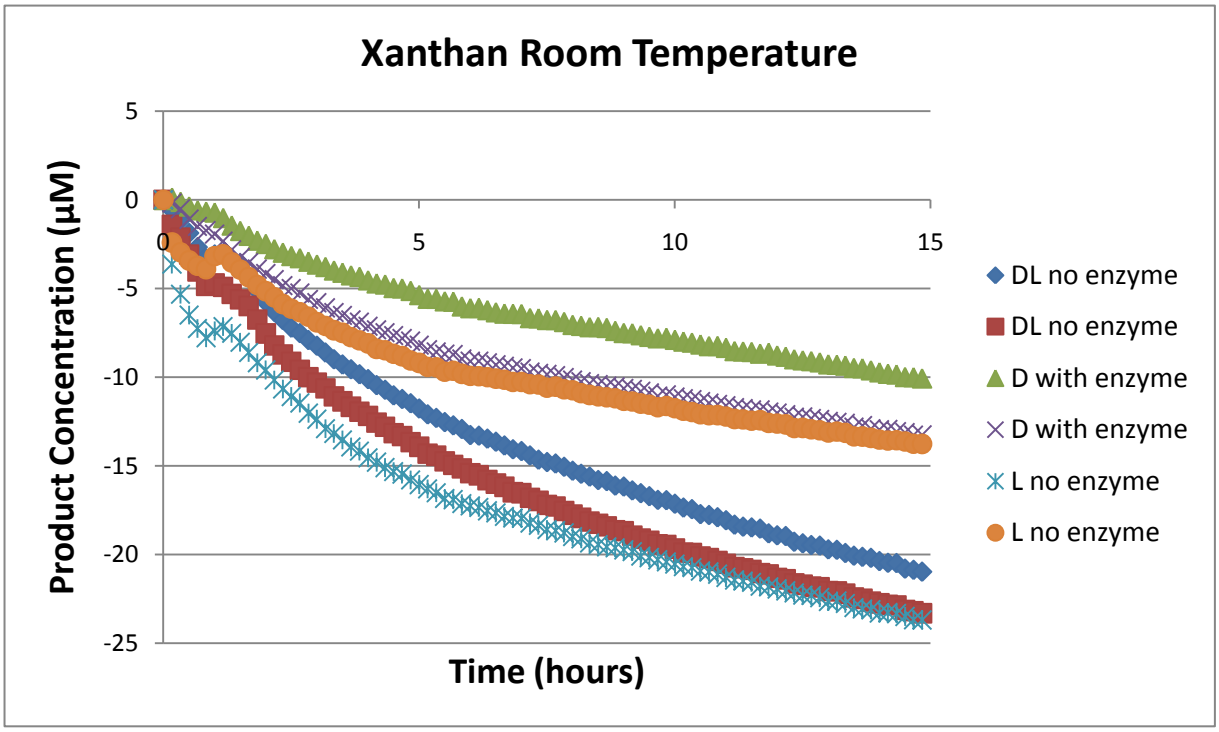
No control samples were made for polyacrylic acid.

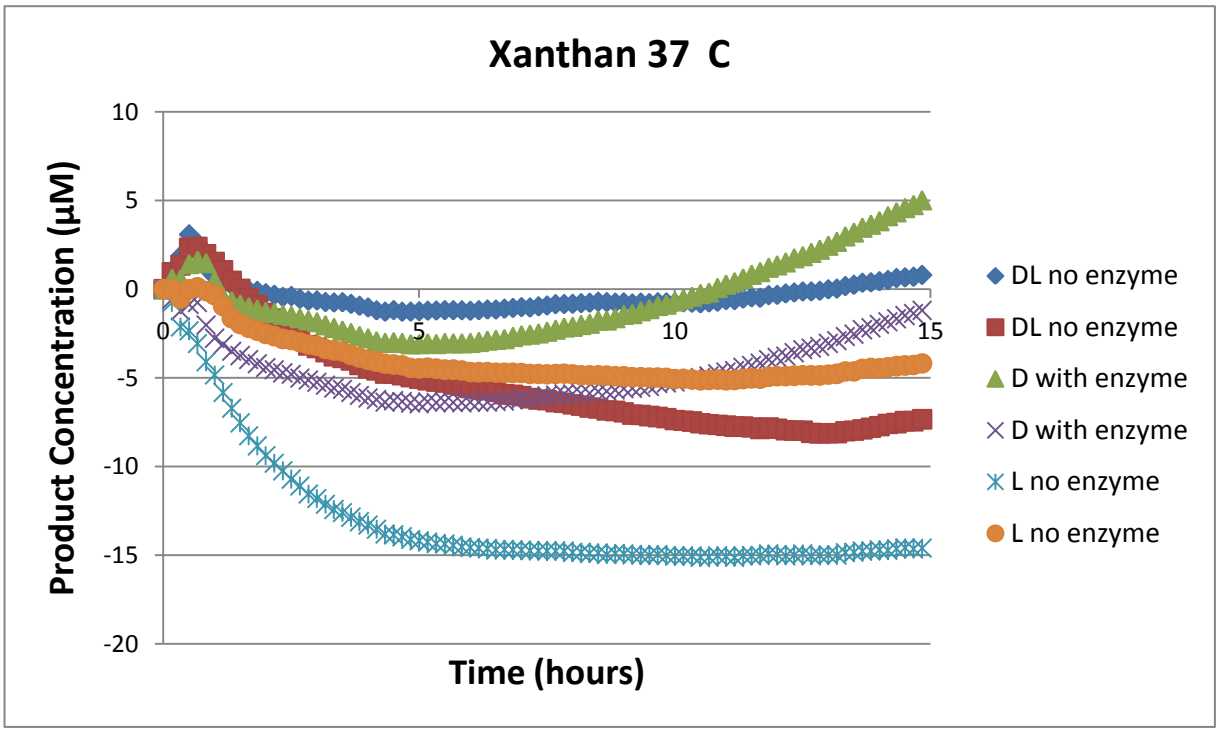
Polyvinylsulfuric acid

Because of the observed precipitation at 10°C in the normal run no control samples were made for that temperature. The other two samples showed negligeble variation (not shown).

Xanthan


Xanthan 10 C
Product Concentration (µM)
Time (hours)
4
2
0
-2
-4
-6
-8
0
5
10
15
DL no enzyme
DL no enzyme
D with enzyme
D with enzyme
L no enzyme
L no enzyme


Xanthan Room Temperature
Product Concentration (µM)
Time (hours)
5
0
-5
-10
-15
-20
-25
0
5
10
15
DL no enzyme
DL no enzyme
D with enzyme
D with enzyme
L no enzyme
L no enzyme



Carboxymethyl cellulose

All control samples showed variation corresponding to less than 1 µmol of product and is thus not shown.