http://dx.doi.org/10.4314/jpb.v8i2.7

Vol. 8 no. 2, pp. 103-114 (September 2011)

http://ajol.info/index.php/jpb

Journal of PHARMACY AND BIORESOURCES

Synthesis of sugars catalysed by microgel conjugated rabbit muscle aldolase in aqueous and aqueous-organic solvents I

Moustapha E. Sesse Koker*

Chemical Laboratory, University of Kent, Canterbury CT2 7NH, United Kingdom
Present Address: *Department of Chemistry, Fourah Bay College, University of Sierra Leone, Mount Aureol, Freetown, Sierra Leone*

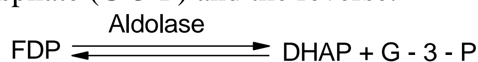
Received 15th July 2011; Accepted 19th August 2011

Abstract
Microgel has been prepared and covalently conjugated to rabbit muscle aldolase and employed to catalyse reactions between dihydroxyacetone phosphate, DHAP natural and non-natural acceptors aldehydes in aqueous, aqueous – organic solvents at ambient temperature. The microgel conjugated enzyme was found to be more stable than the unbound free enzyme and catalysed reactions faster than the latter and in good yield. Reactions took place in aqueous-organic solvents though they were generally slower than in aqueous medium. The generation of a non-natural sugar utilising this microgel enzyme mediated reaction is presented.

Keywords: Microgel-Aldolase; Aldehyde acceptors; Aqueous-organic solvents

INTRODUCTION

The enzyme aldolase normally catalyses the breakdown of fructose 1, 6-diphosphate (FDP) to dihydroxyacetone phosphate DHAP) and glyceraldehydes 3-phosphate (G-3-P) and the reverse.



In biological systems this reaction takes place in aqueous medium and at physiological pH. This property of aldolase has been exploited to carry out organic synthesis.

Rabbit muscle aldolase (EC 4.12.10) is a very available enzyme that catalyses the condensation of DHAP and few close analogues with aldehydes (Whitesides and Wong 1985). This enzyme has also been found to accept a number of aldehydes as substrates and has proved useful in the preparation of a number of rare and unnatural sugars (Durrwachter and Wong 1988, Straub *et al.*, 1990). Bednarski *et al.* (1989) have done studies on aldolase-catalysed reactions of DHAP and various aldehydes in addition to the natural unbound, immobilized or as membrane-enclosed enzymatic catalyst (MEEC) in aqueous and organic co-solvents of up to 20% dimethyl sulphoxide (DMSO) and ethanol. The rate of reaction for some aldehydes in the former solvent system was found to be slightly faster than in the aqueous system.

Davey *et al.* (1989) have investigated microgels as soluble enzyme supports in organic media. Using the enzyme α-chymotrypsin sulphatase and estrase, these

* Corresponding author. *E-mail address*: mesessekoker@yahoo.co.uk Tel: +232-76693810
ISSN 0189-8442 © 2011 Faculty of Pharmaceutical Sciences, University of Jos, Jos. Nigeria.

were conjugated to soluble carboxyl-bearing microgels with water-soluble carboniimide. Enzymatic activity toward several substrates were observed in aqueous –organic and organic solvents. Michaelis-Menten parameters for the enzyme- polymer conjugates were determined and were compared with results from the native enzyme preparation in water and enzyme immobilized on insoluble supports. They found the polymer did not interfere with catalytic properties of these enzymes, and α-chymotrysin exhibited activity in aqueous solutions of methanol and tetrahydrafuran (THF) but not in either of the pure organic solvent; an increase in V_{max} for sulphurase in going from aqueous into methanol solvent and the absence of catalytic activity in esterase in methanol and THF as solvents.

Studies have therefore been undertaken to synthesize non natural sugars by the reaction of DHAP with a non-natural substrate aldehyde catalysed by microgel conjugated rabbit muscle aldolase in aqueous, aqueous – organic and organic solvents. It is the thinking that the microgel conjugated enzyme – aldolase – holds the prospect of

(a) Enhancing the stability of the enzyme relative to the its unbound, unconjugated state in reactions it catalyses
(b) Increase its ability to tolerate non aqueous solvents in reactions meditated by it.

EXPERIMENTAL

Chemicals were purchased from Sigma-Aldrich and were both reagent and analytical grade. Dowex Resins were obtained from British Drug House (BDH) Biochemicals including Aldolase (lyophilised) were purchased from Sigma Company. NMR spectra were recorded on either a JEOL PMX60SI 60 MHz, a JEOL JNM-PS-100MHZ JEOL JNM- GX270 FT NMR 270 MHz using tetramethylsilane (TMS), sodium 2, 2-dimethyl-2-silapentane-5-sulphonate (DSS), or phosphate as internal standard. Fractogel was obtained from Merck Company. Optical rotations were measured with a Bellingham and Stanley Pepol '60'.

High pressure Liquid chromatography (HPLC) was performed on Philips Pye Unicam PU 4010 instrument equipped with a refractometer Model 1109 Refracto Monitor III (range $10x10^6$) a Philips PM 8251 single pen recorder (range 10 mV) or a Philips Pye Unicam PV 4810 Computing Integrator, and a Dynamax Amino Column 4.6 x 250 mm, particle size 8 μm, pore size 60 nm. The refractometer had a circulating water bath at room temperature connected to it.

Enzymatic assays were performed using a Perkin Elmer 124 Double Beam Spectrophotometer of variable wavelength (to 850nm) connected to a power unit with Tungsten and Deuterium lamps, a thermostat bath at 25° and a servo scribe recorder at 10mV full scale deflection (FSD) Doubly distilled water was used in all enzymatic experiments. Moisture was excluded from Enzyme samples by storing them over Silica in the refrigerator at appropriate temperatures.

Preparation of polymer (Microgel)

A. Methylmethacrylate was purified by extraction with 0.1M NaOH, then four times with distilled water and dried over anhydrous sodium sulphate 2-Ethoxyethyl methacrylate, Ethylene glycol dimethacrylate were purified in the same manner. Acrylic acid was purified by distillation at the aspirator pressure. One hundred millilitres of doubly distilled water in a two-neck round bottomed flask was degassed with helium followed by addition of 150mg of sodium lauryl sulphate. The temperature of this solution was brought to 71^0 on an oil bath while stirring and nitrogen gas was passed through the solution. The monomer feed which consists of methyl methacrylate, 2.25 mL, 2-ethoxyethyl methacrylate, 1.5mL, ethylene glycol dimethacrylate, 0.5 mL, and acrylic acid 0.5 mL, and acrylic acid, 0.75 mL, (total 5 mL) was added to the solution, nitrogen gas

bubbled through it for 75 min, the temperature being maintained at 71^0 and with stirring. Ammonium persulphate (initiator 50 mg) was added, the reaction vessel shaken vigorously by hand at interval until a blue opalescence appeared. The inert atmosphere over the reaction was maintained at all times except during the shaking periods. The reaction was continued a bit longer after the blue opalescence first appeared. Quinol (terminator, 50 mg) was added 198 min after addition of initiator.

In a repeat of this reaction, the period between addition of initiator and terminator was 35 min. The solution was centrifuged to remove any solid particles or coagulated matter. The polymer (microgel) was purified by adding HCl (0.1-0.5M) causing precipitation of the product which was recovered by centrifugation and addition of dilute NaOH (0.1M-0.5M) which brings it into solution. This alternate addition of dilute HCl (precipitation) and dilute NaOH (solution) is done several times until the supernatant after centrifugation is quite clear. The product obtained was then stirred with Dowex 2-x8 (20-50µs, 75g) in the hydroxide form for 1 hr. to give a homogenous solution.

Test of purity of polymer. Sephadex G 200-120, (particle size 40-120µ) was swelled in doubly distilled water. This was fed onto a column with a plunger (Phamacia Fine Chemicals) connected to a peristaltic pump, an LKB2238 Bromma UNICORD S II Detector, on LKB Bromma Recorder. Settings of the chromatographic runs were: Chart speed – 0.5 cm/mm; flow rate of solvent (H_2O) 4mL/mm; Full scale deflection (FSD) – 100mV; Absorbance range – 0.5, Time constant 0.5. Used 208nm or 280nm filters in detector. 0.3mL of polymer solution was added on to the column. It gave a single quite symmetrical peak with retention time of 8 min.

Solutions of monomers (e.g. acrylic acid) were applied onto the column to compare their retention times to the polymer. They were clearly different. Samples of polymer (two each of 2mL in a Petri dish) were placed in an oven and weighed periodically until the weights were constant. The concentration of the polymer solution was thus determined. The sample prepared had a concentration of 39 mg/mL, the second batch 19.5 mg/mL.

Monomer feed consisting of the same monomers in the same proportion as in A was added to an aqueous solution of sodium lauryl sulphate (100mg) in a glass soda bottle which has been equilibrated to 70^0 in an oil bath and been purged with nitrogen. Polymerization was initiated by addition of ammonium persulphate, the bottle sealed with its screw cap and shaken vigorously at intervals. A blue opalescence appeared prior to the formation of solid particles 19 min after addition of initiator. Quinol (50 mg) was added after this time to terminate polymerization. The product obtained was purified by adding 0.1MHCl causing precipitation followed by centrifugation and dissolution of the solid in 0.1M NaOH. Further purification by precipitation and dissolution was done twice by 5 M HCl and 2M NaOH respectively. The purified polymer was stirred with Dowex (2-X8, 20- 5 µs, 50g) in its hydroxide form for the resins.

A sephadex column was prepared in doubly distilled water. Polymer (0.3mL) was injected onto the column with the following conditions: Flow rate (H_2O) 4mL/min; chart speed 0.5cm/min.; FSD of recorder 100 mV; wavelength of detector at which run was made – 253nm.

A single symmetrical peak of retention time 9 min was obtained. This was compared with the retention times of methyl methacrylate, 2% acrylic acid under identical conditions and were found to be different.

Assay of aldolase: A solution of aldolase 0.5mg/mL) was made in doubly distilled water. The assay mixture consisted of: 3 mL triethanolamine buffer pH 7.6; 0.3 mg

NADH; 0.1 mL fructose-1, 6- diphosphate (30 mg/mL prepared in H_2O); and 0.02mL GDH/TPI.

Another cuvette containing only triethanolamine pH 7.6 (3mL) both cuvettes are placed in a double beam spectrophotometer set at 340nm. Prepared enzyme solution (0.0mL) was added to the assay mixture, stirred thoroughly, enzymatic activity being determined by the rate of NADH loss time under the following conditions: Chart speed of recorded-1cm/min, FSD 20mv; ambient temperature 26^0(on average).

Conjugation (coupling) of aldolase with polymer. Polymer solution (50 mg 3.5 ml) was made to 5ml with doubly distilled water and the pH adjusted to 6.6 with dilute HCl. Aldolase (5 mg) was dissolved in 5 mL doubly distilled water to which has been added fructose – 1, 6 disphosphate (FDP) tetracyclohexylammoium (TCHA) salt (185.4mg, 20 mmd). This mixture was stirred at 4^0 for 30 min after which the polymer solution was added to it. The enzymatic (aldolase) activity of the reaction mixture was determined, allowed to stir at 4^0 (cold room) for 15 min followed by addition of 1-ethyl -3-(3-dimethylaminopropyl) carbodiimide (EDC)(5mg) and another determination of enzymatic activity. The reaction mixture was then stirred at 4^0 (cold room) for 16hr after which enzymatic activity was again determined.

Purification: The reaction mixture was transferred to an ultrafiltration cell [constructed by the University of Kent's Chemical Laboratory Mechanical Workshop. Two sizes: One of capacity 250mL, the other of 15mL] of capacity 15mL containing an ultrafiltration membrane (DIAFLO® from Amicon Division, WR Grace and Co., Danvers, MA 01923 USA) X M300, 10mm. The reaction mixture was ultrafiltered under nitrogen at 4^0 (cold room) with 0.1M triethanolamme buffer pH 7. The effluent from ultrafiltered polymer bound aldolase was monitored for enzymatic activity. This was done until the enzymatic activity in the effluent was 1% that of the ultrafiltered polymer bound enzyme in the ultra filtration cell, the final activity of the polymer bound enzyme is then determined. Conjugation of Aldolase with polymer was accomplished on a much larger scale by increasing the amounts of reagents proportionately, reaction time same as before with ultra filtration achieved by a cell of 250 mL capacity containing membrane x 300, 62 mm.

Proof of covalent binding of aldolase to polymer. The ultrafiltered polymer bound enzyme was homogenous, of pH7. A sample of this product (0.5mL) was placed in a test tube and the pH adjusted until the polymer falls out of solution - precipitates. The sample was then centrifuged and the supernatant was tested for enzymatic activity. No activity was observed in the supernatant. This indicates all the activity in the polymer bound enzyme was due to enzyme that is covalently bound to polymer. Activity in the supernatant would have indicated that activity (or part of it) in the polymer bound enzyme is not entirely covalent bound.

Assay of aldolase activity from its reaction with fructose 1,6-diphosphate and colorimetric determination of derivatised products. Two test tubes (Experimental and Control) were set up and the following reagents were successively pipetted into them.

	Experimental	Control
Aldolase (0.2mg/mL)	0.02mL	0.02mL
Triethanolamine-hydrazine buffer pH 7.4	1.75mL	1.75mL
Fructose 1, 6-diphosphate	0.25mL	-

Both were incubated at 37^0 for 60mm, and trichloacetic acid (10% w/v) was added.

	Experimental	Control
Trichloacetic acid (10% w/v)	3.0mL	3.0mL
Fructose 1, 6- diphosphate	-	0.25mL

Samples from Experimental and Control were filtered if necessary. NaOH (0.75M, 0.75mL) was added to 1mL of filtered sample in experimental, and 1mL in control and both allowed to stand at room temperature for 1min. 2,4-Dinitrophenylhydrazine (0.1% w/v, 1mL) was then added to each of the experimental and control samples incubated for 10 min at 37^0 (water bath) followed by addition of NaOH (0.75M, 8.25mL) and mixed thoroughly. A spetrophotometric run at 540nm of the resulting solutions (3mL each) was made at ambient temperature and the absorbance noted.

Assay of polymer bound aldolase in water, aqueous-organic and organic solvents by the colorimetric method.

The procedure was the same as immediately above, the only modification being when an aqueous-organic or organic solvent is employed in an assay, the reagents for that particular assay are prepared in the appropriate solvent system. Solvents employed were MeOH, 3:1, 1:1, 1:3 MeOH – H_2O CH_3CN, 3:1, 1:1, 1:3 CH_3CN- H_2O

(i) Dihydroxyacetone phosphate. Ethylcyclo acetal of dihydroxyacetone:

Dihydroxyacetone dimer (5g) was dissolved in 15ml dry ethanol followed by addition of triethylorthoformate (8.5g, 9.6ml). Ammonium chloride (0.25g) was ground, 15ml dry ethanol added to it, heated for 20 min, then added to the dihydroxyacetone solution. The reaction was stirred at room temperature for 4 days after which it was poured into 200ml ether, the ethereal solution washed with water (25 ml) followed by addition of dilute ammonia to make the solution alkaline. After drying over anhydrous sodium sulphate, ether was removed on the rotary evaporator to give an oil which was recrystallised from ethyl acetate 2.54g. ^{1}H NMR (MeOH.d4)δ 3.6 (m, 12H), 1.2(t,6H). The reaction was repeated to obtain more of this product.

(ii) Phosphorylation of ethylcycloacetal of dihydroxyacetone: A solution of $POCl_3$ (12ml, 129mmol) in 85ml absolute pyridine was maintained at -10^0 to -2^0C while ethylcycloacetal of dihydroxyacetone 11.81g, 50mmol) in 85ml dry pyridine was dripped in over 1 hr. The solution was stirred at room temperature for 45 min, then poured into 710 ml of ice cold 0.5M $NaHCO_3$, the reaction mixture being kept at pH7 by addition of HCl. It was then stirred at room temperature for 15 hr followed by degassing 82 ml of magnesia mixture [prepared by dissolving 133.5g $MgCl_2.6H_2O$, 100g NH_4Cl in 500 ml of warm water, ammonium hydroxide added until the solution is alkaline to litmus. After 1 hr the solution is filtered and the filtrate made slightly acid to litmus with HCl. Shortly before use, 15ml of concentrated ammonia per 100ml of mixture are added] was added to precipitate inorganic phosphate (Pi) which was removed by centrifugation after standing at 4^0 for 18hr, (A ^{31}P NMR indicated there was scarcely any Pi present) To the supernatant was added 82 ml of a solution of 40g $BaCl_2$ $.2H_2O$ in 100ml carbondioxide-free water, 2M NaOH added to adjust to pH 8.9. After 18hr at 4^0, the solution was filtered, the barium salt precipitated by addition of 1340ml ethanol to the filtrate, and the resultant mixture kept at O^0 for 9hr. The salt was collected by centrifugation, washed twice with ethanol (80%, then absolute) and ether, and dried in vacuo to give 20.4g of barium salt.

^{1}H NMR (D_2O) δ 2.4(m, 12H), 1.8 (m, 6H) ^{13}C NMR (D_2O, pH4) δ 97.53, 94.53, 63.26, 62.13, 61.09, 57.67. 5.25, 14.5

Analysis: Calcd for C_{10} H_{18} O_{12} P_2 Ba_2: C, 18.0; H, 2.7 found: C: 16.39 H 3.88

Conversion of barium salt acetal to DHAP: The barium salt (1g) was treated with 10ml of Dowex 50 W X 8 H+ in a total volume of 24ml of water. Water was decanted from the resin and this solution (pH1.7) was hydrolyzed at 68^0 for 2.5hr. Determination of

DHAP obtained from the above reaction was accomplished by doing an assay at 340nm

	Experimental	Control
Phosphate buffer pH7.5	1.2ml	1.2ml
Dilute hydrolyzed	1.0ml	-
Water	0.7ml	-
Dilute α GDH (in water)	0.1ml	-
NADH	0.4mg	--

The difference in absorbance in assay mixture before addition and after addition α GDH is noted the change in absorbance Δ Absorbance is in direct proportion to the amount of DHAP present. Initially triethanolanaine buffer was used in determining DHAP but was found to be not as efficient as the phosphate buffer.

Conversion of DHAP to the cyclohexylamine salt. DHAP solution (50 ml), obtained from hydrolysis of the barium acetal (3g) was cooled in an ice bath. Cyclohexylamine in methanol (15%) was added to the mixture until the "PH" was 7.11. The resulting cloudy solution was lyophilized. A sample of the lyophilized product was assayed for the presence and amount of DHAP which was the case.

Reaction of dl-gylceraldehyde and dihydroxyacetone phosphate (DHAP) catalysed by aldolase: A sample of freshly prepared DHAP was adjusted to PH 7.12 18ml of this sample of DHAP (1.04 mmol) was added to dl –glyceraldehyde (90mg, 1.0 mmol) in 3ml doubly distilled water. The mixture was stirred under argon, aldolase (1.23 mg, 12.1 Units) added and stirring continued under argon for 5 hr. Trichloroacetic acid (7%, 3ml) was added and the solution was centrifuged. The supernatant was treated with Dowex 50W x 4 (10g), the resulting solution (pH 1.58) hydrolysed on a steam bath for 10 hr. 2ml of this hydrolysed solution was adjusted to pH 7.35 with 2M NaOH and filtered. An HPLC of the pH-adjusted, filtered reaction mixture was carried out using 4:1 acetonitrile-water. This had a retention time of 5.08 min., which compared favourably to the time (5.13 min) of fructose/sorbose mixture obtained under identical conditions. Co-injection of the reaction mixture and fructose/sorbose mixture gave a single peak of retention time 5.14 min. in the HPLC.

Reaction of dl-glyceraldehyde and dihydroxyacetone phospate (DHAP) catalysed by polymer bound aldolase

A. A sample of freshly prepared DHAP was adjusted to pH 7 with 2M NaOH. 5ml (0.308mmol) of this sample was added to dl-gylceraldehyde (25mg; 0.277mmol) and stirred under argon at room temperature. Polymer bound aldolase (6.6ml, 0.6 Units) was then added and stirring continued at room temperature for 10 hr. Trichloroacetic acid (7%, 4ml) was then added, the solution was centrifuged and the supernatant treated with 3.5g Dowex 50WX4. The resulting solution was hydrolysed on a steam bath for 10 hr. A portion of the hydrolysed solution was adjusted to pH 7 and filtered. HPLC of this filtered mixture was carried out using 4:1 acetonitrile/water its retention time (4.9 min.) comparing to that of fructose (5 min.). A co-injection of the reaction mixture and fructose gave a peak, retention time 5 min.

B. To a solution of DHAP (13.75ml, 1.068mmol) which has been adjusted to pH 6.8 was added dl-glyceraldehyde (90mg, 1.0mmol, the mixture stirred under an atmosphere of nitrogen. Polymer bound aldolase (20ml, 5.67U) was added, and stirring continued under nitrogen at room temperature for 6.5 hr after which the reaction was quenched by the addition of 2ml 7% perchloric acid. The reaction was followed by taking ahquots at intervals, neutralizing them and determining the amount of DHAP. The reaction mixture was centrifuged and the supernatant treated with Dowex 50 X4 – 100 (9.5g). This solution (pH 1.20) was then heated on a steam bath

for 11 hr to hydrolyse phosphate group. The volume of the hydrolysed reaction mixture was 30.6ml. 500µL of hydrolysed reaction mixture was neutralized (IM NaOH) and an HPLC run of this was run in 4:1 acetonitrile-water. This was compared with the HPLC of standard solutions of D– Fructose (5mg/ml), L-sorbose (5mg/ml) and a mixture of D-fructose (L-sorbose (4mg/mL each) The retention time (6min.) of product peak from reaction mixture were identical with that of each of the standards and also by co-injection of reaction mixture and either of the standards. The yield of the reaction was determined by: A HPLC run of 20µL of D-Fructose/L-sorbose standard mixture; that for 20µL of reaction mixture; then a run of reaction mixture and D-Fructose/L-sorbose (10µL each) and a determination of the area under each peak using an integrator. On this basis the yield was 78% averaged over four runs.

Reaction of D-glyceraldehyde and DHAP cyclohexylammonium salt catalysed by polymer bound aldolase. DHAP. Cyclohexylammonium salt (109mg) containing 0.5mmol DHAP and D-glyceraldehye (56mg, 0.62mmol) were dissolved in doubly distilled water (8ml) the mixture being stirred under nitrogen for 1 hr. Polymer bound aldolase (3.5ml, 5.4 Units) was then added, the reaction stirred at room temperature under nitrogen for 4 hr. The reaction was monitored for the disappearance of DHAP during this period. It was quenched by addition of 1ml 7% $HClO_4$ and polymer removed by centrifugation. The supernatant from above was dephosphorylated by treating it with Dowex 50 (H^+ form 6ml) to pH 1.75 followed by heating on a steam bath for 10.5 hr. Total volume of the hydrolysed product was 12.2ml. 500µL of this product was neutralized (IM NaOH) and subjected to HPLC using 20% H_2O – CH_3CN. The retention time (6 min.) of this product was found to be the same as that for D-Fructose.

Reaction of DHAP and propionaldehyde catalysed by aldolase. Crude lyophilised DHAP (370 mg) was dissolved in 2ml 0.2M triethanolamine and adjusted to pH7 with 2 M NaOH. Propionaldehyde (0.2 ml) was added followed by a solution of aldolase (3mg in 2ml) and the reaction was stirred under nitrogen at room temperature for 48 hr. The reaction was monitored for the disappearance of DHAP during this period.

Reaction of DHAP salt and propionaldehyde catalysed by polymer bound aldolase. A DHAP cyclohexylammonium salt (324 mg) containing 0.5 mmole DHAP was dissolved in polymer bound aldolase (33.5ml, 3 Units) This was stirred under nitrogen at room temperature for 20 min. followed by addition of propionaldehyde (40µl), the reaction monitored by determining the decrease of DHAP with time. More propionaldehyde was added at various times after starting the reaction, a total of 610µl being added, as well as polymer bound aldolase (11ml) and some free aldolase (4.6 mg). The reaction was allowed to run for 25 hr. after which it was quenched by addition of 2 ml of 7% $HClO_4$. Polymer was removed after centrifugation of the reaction mixture and the supernatant stored in the fridge.

Enzymatic hydrolysis of product obtained from reaction of propionaldehyde and DHAP. The product (supernatant) obtained in previous reaction (19.4ml) was initially neutralised (1M NaOH), then treated with Dowex 50WX8 (H^+ form) to slightly acidic PH. The solution is purged with nitrogen for 0.5 hr followed by addition of acid phosphatase (6 mg, 2U) the mixture stirred at room temperature for 6 days after which the phosphate enzyme was denatured by heating to 75°. The solution was concentrated then extracted continuously (Sohxlet) with ethyl acetate for 24 hr. The aqueous layer was evaporated at reduced pressure nearly to dryness, then extracted (6x10ml) with boiling

acetone. The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo to yield an oil (152 mg). The residue from the acetone extraction was saved as well (323 mg) ^{1}H NMR of product from organic layer (MeOH, d_4) δ 3.93 (d,d), 3.49 (d,d) 3.2(m, weak), 2.49 (s,), 2.19 (s), 1.37(m, weak), 1.27 (s), 0.98 (t, weak)

RESULTS

The microgel (polymer) having been prepared and purified (Luthra *et al.*, 1987) was conjugated covalently (Davey *et al.,* 1989) to aldolase in 20 millimolar FDP by treating the reaction mixture with 1-ethyl-3-(3-N,N-dimethylamino) carbondirmide (EDC). The generated microgel-aldolase was purified by ultrafiltration. Test of covalent conjugation to the enzyme was ascertained by adjusting the pH with O.1 M HCl until the polymer precipitates, the sample centrifuged and supernatant tested for activity which was found to be negative. Activity of the conjugated enzyme was tested by employing it to catalyse the breakdown of FDP to its component products. The reaction was followed spectrophotometrically at 540 nm, enzymatic activity being in direct proportion to absorbance (Bergmeyer 1974). The microgel aldolase in a 0.1M ethanolamine buffer pH7 – 7.5 was stored at 4º and was stable for five days without any appreciable loss of activity, but a slight decrease over a twenty day period.

Reaction of FDP as the tetracyclohexylammonium salt, was carried out in water, and H_2O – MeOH mixtures catalysed by microgel – aldolase. The extent of the reaction and hence the activity of the microgel-enzyme was measured by the absorbance at 540 nm. It was observed that the activity of the microgel bound enzyme decreased with increase in the proportion of methanol in the solvent mixture (Table I).

It was found that absorbance and hence the extent of the reaction, was proportional to the amount of microgel bound enzyme for solvent systems containing a higher proportion of water (though it was not necessarily so for systems containing higher proportions of methanol. When the trisodium salt of FDP and microgel bound aldolase were employed with the same mixtures of water – methanol, an increase in the absolute values for absorbance was observed but the trend was the same: absorbance and hence rate of reaction, decreased with increase in the proportion of methanol in the solvent mixture (Table II). Similar trends were observed in the absorbance when mixtures of water and acctonitrile solvent systems were employed in the apway (Table III).

Dihydroxyacotone phosphate (DHAP) was prepared (Effenberger and Straub 1987) and its amount and yield determined in phosphate buffer (Colbran *et al.,* 1967). This was followed by reaction of DHAP and D, L-glyceraldehye catalysed by microgel bound aldolase at room temperature under argon for 6.5 hrs. The product obtained was dephosphorylated by treating it with Dowex 50 w x 4 followed by heating on a steam bath for 10 hr. HPLC (4:1 CH_3CN / H_20) of the resulting product gave a retention time of 4.9 min. which compared favourably with that of fructose/sorbase mixture (5 min.). Co-injection of this product and fructose/sorbase mixture gave a single peak of retention time 5 min. The yield of this product determined by HPLC(4:1 CH_3CN / H_20) was 80%.

Condensation of propronaldehyde with DHAP mediated by polymer bound aldolase was carried out at room temperature. The reaction was monitored by determining the amount, of, hence the loss of DHAP with time. Reaction went to 80% completion after 30 hours. When the same reaction was repeated with free enzyme, it went to 80% after 38.5 hours and to completion after 48 hours. The DHAP samples employed in previous reactions were either as an aqueous solution or in the lyophilised state. It was

decided to convert DHAP into the cyclohexylamine salt which would make it convenient to store and possibly enhance its solubility in organic solvents. This was accomplished by treating DHAP with 15% cyclohxylamine in methanol adjusted to neutral "pH" in an ice bath followed by lyophilisation. The yellow to light brown product though a solid must be stored below room temperature. When dissolved in water, methanol and ethanol, this salt was found to decompose with time, the rate being slowest in water than in methanol and highest in ethanol. DHAP cyclohexylamine salt having been prepared, was reacted with D-glyceraldehyde catalysed by polymer bound aldolase (Scheme 1).

It was run on 0.5 millimolar scale with the aldehyde added in slight excess. The D-fructose phosphate generated was dephosphorylated to D-fructose in 75% yield determined by HPLC. This reaction was faster (4 hours) than similar reaction with dl-glycerldehyde (6.5 hours). A similar reaction mediated by polymer bound enzyme was effected between propionaldehyde and DHAP cyclohexylamine salt on 0.5 millimolar scale (Scheme II).

It was similarly monitored by determining the amount of DHAP salt in the reaction mixture with progression of the reaction. The product obtained was reacted with acid phosphate to hydrolyse the phosphate group. Workup of the reaction mixture by soxhxlet extraction gave an organic fraction and a solid residue. Proton NMR or product from the organic layer showed peaks which gave some confirmation of the presence of the product but they were not as intense at δ0.98 (triplet), 1.38 (multiplet) 3.2 (multiplets), two sets of peaks (doublet of doublets) at δ 3.5 and 3.92. These latter peaks are of equal intensity in the integrated spectrum and integrated for more than one proton.

An earlier reaction of DHAP salt and propionaldehyde did not go to completion after 23 hours, even after addition of unbound enzyme after this period.

Table I

Solvent System	Absorbance (Δ Abs)
H_2O	0.66
H_2O/MeOH 3:1	0.19
H_2O/MeOH 1:1	0.02
MeOH	0.08$^+$

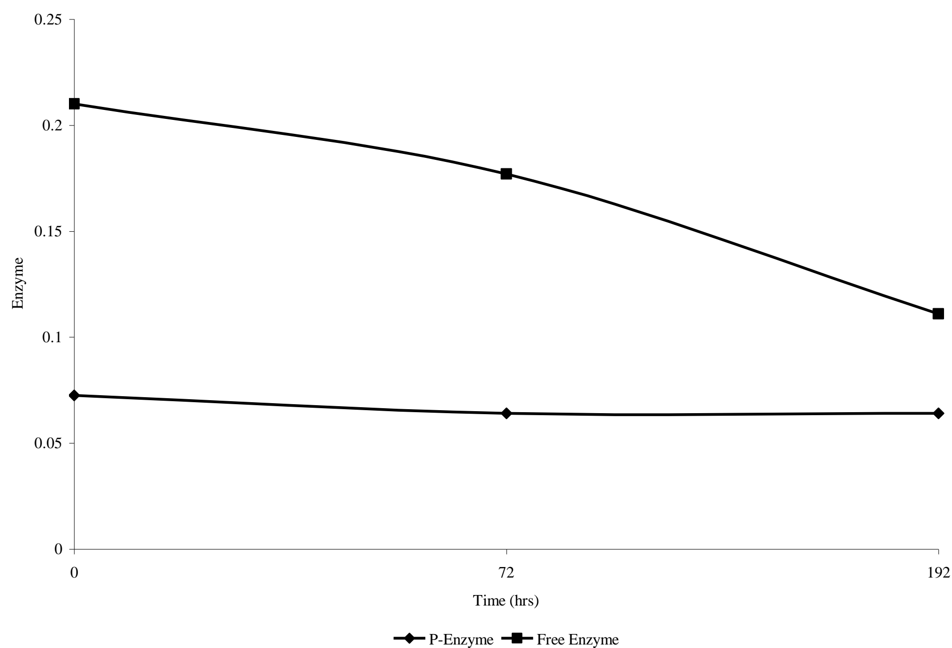
+ Best value obtained. Run was separate from first four entries.

Table II

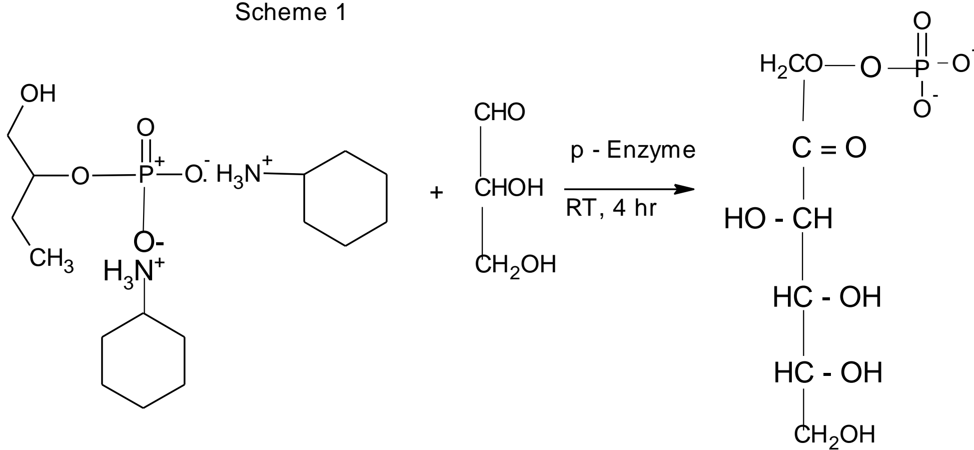
Solvent System	Absorbance (Δ Abs)
H_2O	1.42
H_2O/MeOH 3:1	1.24
H_2O/MeOH 1:1	0.185
H_2O /MeOH 1:3	0.08
MeOH	0.04

Table III

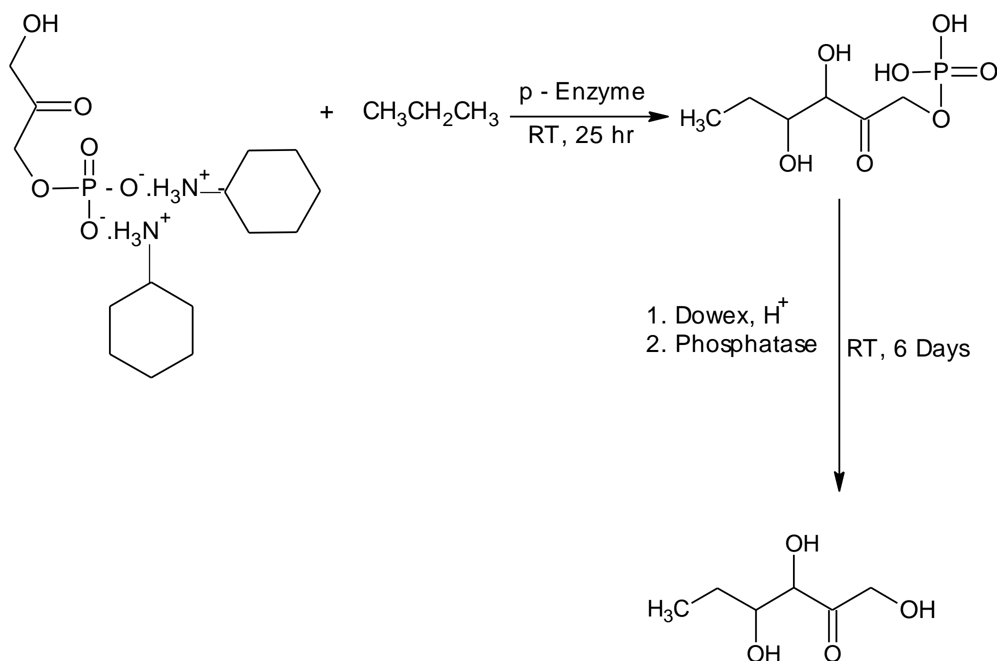
Solvent System	Absorbance (Δ Abs)
H_2O	1.35
H_2O/CH_3CN 3:1	0.15
H_2O/CH_3CN 1:1	0.095
H_2O /CH_3CN 1:3	0.025
CH_3CN	0.025



Scheme 1



Scheme II



DISCUSSION

Davey *et al.* (1989) employed EDC as an agent in the coupling of chymotrypsin to microgel. In this study the use of EDC led to the formation of microgel coupled aldolase with low activity. This necessitated adding 20 millimolar of FDP in the coupling reaction which greatly enhanced the activity of the resulting product. The rationale was with FDP in solution and it also being a natural substrate for aldolase, it would occupy its active site thus preventing any adverse effects of EDC or other reagents during the coupling reaction of microgel to aldolase.

The microgel (polymer) bound enzyme was found to be more stable in 0.1M triethanolamine buffer pH 7-7.5 than the native enzyme in the same medium, thus indicating the microgel has enhanced the stability of the enzyme with respect to the reactions it catalyses. It catalysed the breakdown of the natural substrate FDP in aqueous as well as aqueous – organic solvent though the extent of reaction was found to be slower in the latter solvent systems. The extent of reaction was also found to be different for different salts of the natural substrate FDP.

Initially the condensation reaction between DHAP and propionaldehyde did not go to completion in part because a large excess of propionaldehyde was added at the beginning of the reaction. It is however worth noting the time it took this reaction to go to 80% completion was shorter (30 hrs) for microgel bound aldolase than for the free unbound aldolase (38.5 hrs).

With the generation of the cyclohexylamine salt of DHAP its stability in organic solvents was enhanced relative to the free DHAP. Reaction of this salt with D-glyceraldehyde mediated by polymer bound enzyme yielded D-fructose in good yield (75%), it occurring within a shorter period (4 hrs) than on earlier condensation reaction with D.L$^-$ glyceraldhyde and DHAP mediated by the same microgel-enzyme (6.5 hrs).

Reaction of propionaldyde and DHAP cyclohexylamine salt yielded a condensation intermediate product that was dephosphorylated enzymatically by phosphatase to yield the product 2-keto-1,3,4-

trihydrooxy hexane, which was identified from the proton NMR data.

Conclusion. Microgel conjugated aldolase has been shown to catalyse reactions in aqueouos, aqueous-organic solvents and to a lesser extent pure organic solvents. It has been found to be more stable with regards to activity than unbound free aldolase at room temperature and effects reactions relatively faster. The DHAP cyclohexylamine salt holds the promise of enhancing reactions in pure organic solvents. It also has been shown to catalyse the reaction of DHAP and non natural substrate acceptor aldehydes.

Acknowledgements

This work was supported by a grant from the United Kingdom Science and Engineering Research Council to the University of Kent at Canterbury and for a Research Fellowship. Funds for assistance with preparation of this work for publication from Fourah Bay College, University of Sierra Leone is also appreciated.

REFERENCES

Bednarski, M. D.; Simon, E. S.; Bisdhofberger, N.; Fessner, W. D.; Kim, M. J.; Lees, W.; Saito, T.; Waldman, H..; and Whitesides, G. M. (1989). Rabbit Muscle Aldolase as a Catalyst in Organic Synthesis *J. Am. Chem.Soc.* III, 627 – 635

Bergmeyer, H. U., 1974 Methods of Enzymatic Analysis 3rd ed. Vol. III, New York p. 44, 1104.

Colbran, R. L.; Jones, J. K. N.; Matheson, N. K.; and Rozema, I. (1967). *Carbohyd. Res*. 4, 355-360

Davey, J. P.; Pryce, R. J. and Williams, A. (1989). Microgels as Solubule Enzyme Supports in Organic Media. *Enzyme Microb. Technol.* II 657 – 661.

Durrwachter, J. R. and Wong, C. H. (1988) Fructose 1, 6 Diphosphate Aldolase Catalysed Stereodetective Synthesis of C – Alkyl and N – Containing Sugars: Thermodynamically Controlled C–C Bond Formation *J. Org. Chem.* 53 4175 – 4181

Effenburger, F. and Straub, A. (1987) A novel convenient Preparation of dihydroxyacetone phosphate and its use in enzymatic aldol reactions *Tetrahedron Lett*. 28 1541 – 1544.

Luthra, A. K.; Williams, A. and Pryce, R. J. (1987) Microgels as supports for a - chymotrypsin *J. Chem. Soc Perkin Trans* II 1575 – 1578.

Straub, A.; Effenburger, F. and Fishcher, P. (1990). Aldolase-catalysed C–C bond formation for stereoselective synthesis of nitrogen-containing carbohydrates *J. Org. Chem.* 55, 3926 – 3932

Whitesides, G. M. and Wong, C – H (1985). Enzymes as Catalysts in Synthetic Organic Chemistry *Angew. Chem.* Int. Ed. Engl. 24, 613 – 638.