THE ENZYMES OF THE REDUCTIVE PENTOSE PHOSPHATE PATHWAY OF A GREEN ALGA

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ABSTRACT

As many metabolic pathways are postulated to be catalysed by multi-enzyme complexes, an attempt was made to detect their functioning in the reductive pentose phosphate pathway (RPPP) of *Scenedesmus obliquus*. This entailed isolating the enzymes most likely to be in a complex, raising antisera against them and using the antisera to test for the presence of these enzymes in a complex. Investigation of the kinetic behaviour and quaternary structure of the enzymes may give some evidence for their functioning in a complex. Ultimately, it was hoped that evidence of channelling of intermediates within these complexes would be obtained.

Five enzymes of the RPPP, glyceraldehyde 3-phosphate dehydrogenase, phosphoribulokinase, ribulose 1,5-bisphosphate carboxylase, fructose 1,6-bisphosphate aldolase and phosphoglycerate kinase, were extracted from the alga and purified to apparent homogeneity, as judged from SDS-polyacrylamide gel electrophoresis (PAGE). The chromatography techniques used in the purification were ion exchange, gel filtration, dye-ligand and hydrophobic interaction.

Antisera were raised against these enzymes, and the preparations found to be specific for their corresponding antigens by western blotting.

Whilst purifying aldolase, three isozymes were separated. The isozyme profile in the crude extract was dependent upon the nature of the growth conditions employed. The most basic isozyme (aldolase-3), the activity of which constituted approximately 75% of the total aldolase activity in heterotrophically grown alga, accounted for less than 25% of the total activity in the autotrophically grown alga. This isozyme was most likely involved in glycolysis and localised in the cytoplasm. The other two isozyme activities (aldolases -1 and -2) together, accounted for less than 25% of the aldolase activity in heterotrophic extracts, but provided over 75% of the activity in autotrophic extracts. These isozymes could be the chloroplast fructose 1,6-bisphosphate and sedoheptulose 1,7-bisphosphate aldolases.

The molecular weight of the subunits of all three aldolase isozymes was determined by SDS-PAGE to be 40 000. The specific activities of aldolases -2 and -3 were estimated to be 5.5 and 14.9U (mg protein)$^{-1}$,

respectively. Aldolase-2 was more susceptible to thermal denaturation than aldolase-1.

The molecular weight of the phosphoglycerate kinase (PGK) determined by SDS-PAGE was 45 000, and by gel filtration it was 38 000. Hence, PGK was a monomer.

Gel filtration of algal extract suggested that PGK might be present in a multi-enzyme complex.

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LIST OF ABBREVIATIONS

Abbreviation	*Description*
bisPGA	glycerate 1,3-bisphosphate
DHAP	dihydroxyacetone phosphate
DTT	dithiothreitol
FBP	fructose 1,6-bisphosphate
FPLC	Fast Protein Liquid Chromatography
G3P	glyceraldehyde 3-phosphate
G3PDH	glyceraldehyde 3-phosphate dehydrogenase
LDH	lactate dehydrogenase
mpte	mercaptethanol
Mr	molecular weight
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
PGA	glycerate 3-phosphate
PGK	phosphoglycerate kinase
PK	pyruvate kinase
PRK	phosphoribulokinase
RuBP	ribulose 1,5-bisphosphate
RuBPC	ribulose 1,5-bisphosphate carboxylase
RPPP	reductive pentose phosphate pathway
R5P	ribose 5-phosphate
SBP	sedoheptulose 1,7-bisphosphate
U	unit of enzyme activity

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INTRODUCTION

Photosynthesis

Green plants and certain bacteria are able to absorb the energy of sunlight, and subsequently convert it into chemical energy, which they use to synthesise organic compounds from atmospheric carbon dioxide. This light driven anabolism of carbon dioxide, together with the capture of light energy, is termed photosynthesis. In all plants the oxidisable substrate for the photosynthetic process is water (see review by Foyer (1984)).

Nearly all the chemical energy, organic compounds and food that enters into any ecosystem on earth, is provided by photosynthesis (Björkman, 1975). The total amount of carbon introduced into carbohydrates by photosynthetic organisms is estimated to be approximately 2 x 10^{11} tonnes/year (Hoober, 1984). As a result, all biological activity is ultimately limited by how well photosynthesis is able to operate in all diverse environments that exist. It is therefore very important to know what the environmental restraints and the adaptive restraints of photosynthesis are, as well as to understand the

various mechanisms that underlie photosynthetic adaptation and how they work (Björkman, 1975).

The principal products of photosynthesis are glycolate, triose phosphates and 3-phosphoglycerate (PGA) (Dennis and Miernyk, 1982) and it takes place in three phases (Hoober, 1984):

1. Chlorophylls and other chloroplast pigments absorb light energy, resulting in the production of electronically excited molecules.
2. The excited electrons participate in the primary photochemical reactions, whereby the light energy is converted into ATP and NADPH.
3. The ATP and NADPH generated in the light reactions are used to reduce carbon dioxide to form organic compounds.

Chloroplasts

In all higher plants and certain algae, photosynthesis occurs in the chloroplasts (Steinbeck, 1981). They are the primary source of carbon precursors, reducing power and ATP in plant cells (Dennis and Miernyk, 1982), all of which are generated by photosynthesis (Werden *et al.*, 1972). Chloroplasts are surrounded by a continuous, rather fragile outer membrane. An inner membrane system encloses the internal compartment. Inside the latter and often connected to the inner membrane are flattened, membrane surrounded vesicles called

thylakoids, which are usually arranged in stacks called grana. The thylakoid membranes contain all the photosynthetic pigments of the chloroplast and all the enzymes required for the solar energy-trapping reactions. The fluid in the compartment surrounding the thylakoid sacs is called the stroma and is thought to contain most of the enzymes required for the reactions in the reductive pentose phosphate pathway (RPPP) (see Lehninger (1982)). The chloroplast also contains its own complement of DNA and RNA polymerases, ribosomes and DNA (Steinbeck, 1981). Proteins needed, but not synthesised in the chloroplast, are coded for by the nuclear DNA, synthesised in the cytoplasm and transported into the chloroplast (Weisbeek *et al.*, 1989)

The Reductive Pentose Phosphate Pathway

The RPPP is the biochemical pathway which fixes carbon dioxide (Bassham *et al.*, 1954; Bassham and Calvin, 1957), and is present in all photosynthetic eukaryotic cells (Norris *et al.*, 1955; Anderson and Fuller, 1967). It is comprised of 13 enzyme catalyzed reactions (Fig. I.1), and has three fundamental phases (see review by Foyer (1984)):

1. Carboxylation:

   Carbon dioxide is fixed into the acceptor substrate, ribulose 1,5-bisphosphate (RuBP) to yield two molecules of PGA.

2. Reduction:

   Using ATP, PGA is converted to glycerate 1,3-bisphosphate (bisPGA), which is then reduced by NADPH to glyceraldehyde 3-phosphate (G3P). For continued carbon fixation, the triose

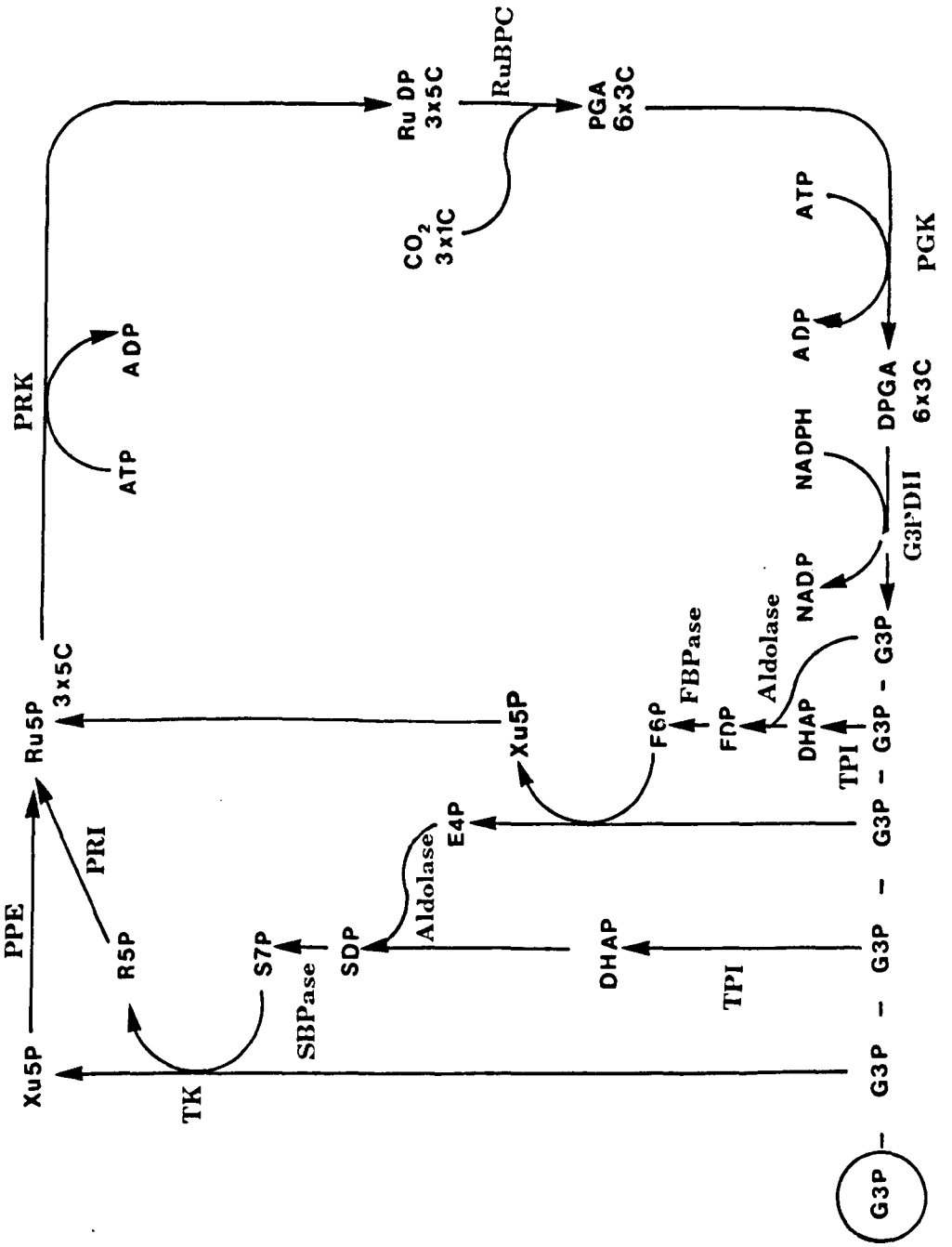


Figure I.1 The reductive pentose phosphate pathway in C_3 plants

phosphates, G3P and its isomer dihydroxyacetone phosphate (DHAP), must either be exported from the chloroplast in exchange for inorganic phosphate or be further metabolized in the chloroplast.

3. Regeneration:

   Five out of every six molecules of triose phosphate formed are rearranged in an array of complex interconversions, involving nine enzymes to regenerate three molecules of RuBP.

The reactions of the RPPP were completely mapped out by means of substrates labelled with radio isotopes (Bassham *et al.*, 1954). One turn of the RPPP results in the carboxylation of three molecules of RuBP, leading to six molecules of PGA, which are then reduced to six molecules of triose phosphates using six molecules of ATP and NADPH each. Five triose phosphate molecules are then reconverted to three RuBP molecules using three molecules of ATP (Bassham, 1972).

When the rate of export of triose phosphate is limiting, fructose 6-phosphate is converted via glucose 6-phosphate to other carbohydrates, including starch and cellulose. Triose phosphates are also converted into glycerol phosphate for onward synthesis of lipids, or transformed into various amino acids (Bassham, 1972).

Under steady-state conditions, only the reactions catalysed by ribulose 1,5-bisphosphate carboxylase (RuBPC) (EC 4.1.1.39), phosphoribulokinase (EC 2.7.1.19) (PRK), fructose 1,6-bisphosphatase (EC 3.1.3.11) and sedoheptulose 1,7-bisphosphatase (EC 3.1.3.37) (Fig.

I.1) are accompanied by a large decrease in free energy, and as such, are virtually irreversible and have a role in metabolic regulation. The rest of the reactions are reversible and are not regulated (Bassham and Krause, 1969). The metabolites between the carboxylation and the sedoheptulose bisphosphatase reaction tend to be able to diffuse from the chloroplast, whilst the rest, except for the pentose monophosphates, are retained (Bassham and Kirk, 1968).

Most of the enzymes operating in the RPPP, are common to glycolysis and a few other carbohydrate pathways in animal, plant and microbial cells (Fuller and Hudock, 1967). The exceptions are PRK, RuBPC and NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (EC 1.2.1.12), which are considered to be exclusively associated with the RPPP and are thus localised solely in the chloroplasts (Latzko and Gibbs, 1968). When a particular enzyme activity is present in more than than one subcellular location, the various forms are termed isozymes (Scopes, 1969).

Regulation

Due to the absence of compartmentation within the chloroplast, the enzymes of the RPPP intermix with enzymes of the oxidative pentose phosphate pathway and glycolysis, and as such the chloroplast must possess mechanisms whereby opposing synthetic and degradative pathways can be regulated (Buchanan, 1980). The principal and ultimate regulator of carbohydrate metabolism is light, which activates

selected biosynthetic enzymes, whereas certain degradative enzymes are de-activated (Macdonald and Buchanan, 1987). Light activation appears to occur not by a direct effect of light on the modulated enzymes, but by a more complex process, the primary light acceptor being a pigment, probably chlorophyll in the photochemical apparatus (Anderson, 1975). Regulatory proteins, such as thioredoxins (Buchanan, 1981), then function as a link between the pigments and the regulated enzymes (Buchanan, 1980). Thioredoxins are small ubiquitous redox proteins possessing groups of two cysteine residues in close proximity, which can be reversibly oxidised to form a disulphide bridge (Rowell *et al.*, 1988). Available evidence suggests that the activation/de-activation results from the cleavage/ligation of sulphydryl bonds on the enzyme protein (Anderson, 1975). Sulphydryl groups play an important role in substrate and co-factor binding and also participate as acyl acceptors (Hatch and Turner, 1960). The alkalization of the stroma (Heldt *et al.*, 1973) and the import of magnesium ions into the stroma (Lin and Nobel, 1971), which create optimum conditions under which enzymes can operate, are two other mechanisms by which light can exert its influence on carbohydrate metabolism (Leegood and Walker, 1980).

Glyceraldehyde 3-Phosphate Dehydrogenase

G3PDH catalyzes the only reductive reaction of the RPPP (Wolosiuk and Buchanan, 1976). In photosynthetic tissues, it has two major functions: the involvement in carbohydrate breakdown by glycolysis; and the photosynthetic assimilation of CO_2 (O'Brien and Powls, 1976).

The chloroplast in *S. obliquus* contains two forms of G3PDH (O'Brien *et al.*, 1976). A high molecular weight form, with low activity, which uses NADH as its preferred coenzyme and a lower molecular weight form, with high activity utilising NADPH as its preferred coenzyme. In the presence of bisPGA or NADPH, the NADPH-dependent activity is enhanced considerably with an accompanying decrease in NADH-dependent activity. This activation is very rapid and independent of protein synthesis (O'Brien *et al.*, 1976). The bisPGA activation has only been detected in *Chlorophyta* (O'Brien and Powls, 1975) and has been shown to be reversible in *S. obliquus*. It is likely that *S. obliquus* synthesises a single G3PDH, and that this protein adopts a particular configuration depending upon the ligands it encounters (O'Brien *et al.*, 1976).

Phosphoribulokinase

In the last step of RuBP regeneration (see review by Foyer (1948)), PRK catalyses the ATP-dependent phosphorylation of ribulose 5-phosphate to produce RuBP, the primary acceptor of carbon dioxide in all photosynthetic organisms (Porter *et al.*, 1986). It is found to exist both as a soluble enzyme and loosely bound to chloroplast membranes (Fischer and Latzko, 1979). PRK is rapidly activated upon illumination of intact chloroplasts and rapidly deactivated upon return to darkness (Laing *et al.*, 1981). The specific activity of PRK is very high, but its affinity for its substrates appears to be low (see review by Foyer (1948)).

Ribulose 1,5-Bisphosphate Carboxylase

RuBPC is the most abundant enzyme on earth (see (Lehninger, 1982)). This protein has two distinct kinds of non-covalently linked subunits which differ in molecular weight and amino acid composition (Rutner and Lane, 1967). The larger subunits form the catalytic entity (Nishimura and Akazawa, 1974) and are bound non-covalently to the RuBPC large subunit binding protein during the assembly of RuBPC in the chloroplast (Musgrove and Ellis, 1986). The smaller subunits may have a regulatory role (Nishimura and Akazawa, 1973; Takabe and Akazawa, 1973). The two subunits are synthesised at separate locations in the cell (Criddle *et al.*, 1970).

RuBPC catalyses the covalent insertion of carbon dioxide and the simultaneous cleavage of RuBP-carbon dioxide complex to yield two molecules of PGA, one of which bears the introduced carbon in its carboxyl group (see Lehninger, 1982)). RuBP is so reactive, that the carboxylation takes place without an added energetic co-factor (Bassham, 1972). This reaction is a relatively complex reaction utilising a substrate (carbon dioxide) which occurs at low concentrations in the stroma (Bassham and Krause, 1969). Oxygen is a mutually competitive substrate with carbon dioxide for RuBPC. Oxygenation of RuBP produces one molecule each of PGA and 2-phosphoglycolate (Somerville and Ogren, 1982). This oxidation is probably the principal component of photorespiration (Bassham, 1972). A soluble enzyme, RuBPC activase, is required for the activation of RuBPC (Ogren *et al.*, 1986).

Aldolase

In the chloroplast, aldolase (EC 4.1.2.13) participates in two different reactions of the RPPP (see review by Foyer (1948)). It catalyzes the reversible condensation of the aldehydes G3P or erythrose 4-phosphate with DHAP to give the six carbon fructose 1,6-bisphosphate (FBP) or the seven carbon sedoheptulose 1,7-bisphosphate (SBP), respectively (see review by Devlin and Barker (1971)). These reactions are freely reversible and are not regulated (see review by Foyer (1948)). The aldolase activity present in the chloroplast is in excess of that required for maximal rates of the RPPP (Murphy and Walker, 1981).

Phosphoglycerate Kinase

The monomeric phosphoglycerate kinase (PGK) (EC 2.7.2.3) (Krietsch and Bücher, 1970) catalyzes the transfer of an energy-rich phosphate group, from ATP to PGA (Scopes, 1973), and in the process, utilises two-thirds of the total ATP requirement of the RPPP (see review by Foyer (1948)). Unlike most kinases, PGK catalyses a freely reversible reaction (Latzko and Kelly, 1979), the equilibrium of which is essentially independent of pH and favours the synthesis of ATP. The utilisation of PGA is favoured by a high ratio of its substrates, MgATP and PGA to its products, MgADP and bisPGA (Hers, 1952; Larsson-Raznikiewicz and Malmström, 1961; see review by Foyer (1948))

which occurs during photosynthesis (Scopes, 1973). Its mode of action is consistent with a rapid equilibrium random mechanism (Larsson-Razniekwicz and Arivdsson, 1971). PGK has one binding site for (bis)PGA and a second site for anions, especially sulphate (Wrobel and Stinson, 1978). It also contains at least two nucleotide binding sites, one presumably binding to MgATP and the other to MgADP (Larsson-Razniekwicz and Arivdsson, 1971).

Algae

A unicellular green alga was chosen as the source of enzymes of the RPPP. Many similarities exist between the metabolism of higher plants and the unicellular green algae and these algae can be maintained easily under laboratory conditions (Klein *et al.*, 1983).

Algae are a heterogenous group of cryptogamic plants ranging from unicellular, through colonial, filamentous, siphonaceous forms to the complex larger seaweeds. They have a long fossil history, some of them probably being among the first photosynthetic plants (Round, 1965). Many algae capable of photosynthesis are also able to assimilate preformed organic substances and are thus able to grow in the absence of light or carbon dioxide (Griffiths, 1965). As primary producers, algae are dominant in aqueous environments, whereas as heterotrophs they are in competition with the rest of the microbial world, for whom larger numbers, smaller size and a higher metabolic

speed and versatility are overriding advantages (Droop, 1974). The number of chloroplasts per algal cell ranges from one to over a hundred in some large celled species (Hoober, 1984).

Scenedesmus obliquus possesses a single, relatively large chloroplast per cell (Graham and Smillie, 1971) and it too can be grown under both heterotrophic and autotrophic conditions, whilst maintaining a fully functional chloroplast (O'Brien and Powls, 1975). It is also relatively easy to produce mutants (R. Powls, personal communication). Its main disadvantage is its extremely tough and elastic cell wall, which requires the use of considerable force for its disruption, thus making it extremely difficult to obtain intact chloroplasts (Graham and Smillie, 1971).

Multi-Enzyme Complexes

The concept of a living cell composed of an aqueous solution uniformly dispersed with enzymes and metabolites is being gradually abandoned in favour of a viewpoint which pictures different, but functionally related, enzymes being bound together by molecular forces into a highly organised structure (Ginsburg and Stadtman, 1970).

A multi-enzyme complex is an aggregate of different but functionally related enzymes bound together by hydrophobic and electrostatic forces, as well as hydrogen bonds, into a highly organised structure.

These interactions are weak, which endow upon these complexes the ability to engage in association-dissociation processes, which may play an important role in both catalysis and in the regulation of enzymic reactions (Ginsburg and Stadtman, 1970; Keleti *et al.*, 1977).

Significance

The existence of multi-enzyme complexes may have a three-fold beneficial role in cellular metabolism (Welch, 1978; Srere, 1987):

1. The clustering of the component enzyme moieties may produce entities that have intrinsic catalytic properties superior to those of the separate enzymes.
2. As a result of complexing there could be the channelling of intermediate metabolites from one constituent enzyme to the next. Channelling spares the limited solvation capacity of cellular water and enables a high concentration of substrates to be achieved with a relatively low number of substrate molecules. It also reduces the likelihood of an intermediate metabolite being diverted into a less useful process as well as ensuring the preservation of those metabolites that are unstable in an aqueous environment.
3. The juxtaposition of relevant enzymes would result in hastening the enzymic reaction by reducing the time it takes for an intermediate metabolite to diffuse from one active centre to the next. Moreover, nascent products may initially be in an excited state, so, if the product could be transferred to the next active

centre before this energy was dissipated to the environment this extra energy could be utilised in providing all or part of the activation energy required for the next step.

Objectives

There is a dearth of information on the existence of such co-ordinated enzyme complexes in the RPPP.

The demonstration that well known soluble metabolic pathways, such as those for the synthesis of fatty acids (Lynen *et al.*, 1968) or the decarboxylation of pyruvate (Reed and Oliver, 1968) are each catalyzed by a specific aggregation of enzymes, has lent impetus to the idea that a significant degree of organisation may exist in other apparently soluble multi-enzyme systems (Reed and Cox, 1966; Mowbray and Moses, 1976). The description of enzymes as being 'soluble', refers to the ease with which they could be extracted using aqueous solutions and does not predict that they occur in a dissolved state in the cell (Arnold and Pette, 1968).

Moreover, the reported occurrence of complexes between aldolase and G3PDH (Kálmán and Boross, 1982), or triose phosphate isomerase (EC 5.3.1.6) (Salerno and Ovádi, 1982) and PGK and G3PDH (Malhotra *et al.*, 1987), in the closely related glycolytic pathway, points to the

distinct possibility that such protein-protein interactions might exist between the constituent enzymes of the RPPP. Consequently the discovery, by members of this research group, of the presence of a multi-enzyme complex in the RPPP containing the activities of both PRK and G3PDH (Lazaro *et al.*, 1986; Nicholson *et al.*, 1987) has been an added incentive in the search for other complexes in this pathway.

The aims of this project were: To search for other multimeric complexes in the RPPP and then to investigate their structural relationship with the G3PDH-PRK complex.

It was necessary to first extract and purify each of the enzymes thought most likely to be present in a complexed state and then to investigate their kinetic behaviour and quaternary structure.

It also entailed raising antisera to each of the enzymes in order to look for complexes in crude cell extracts, for possible cross reaction between the enzymes and to study the dissociations of these complexes and their accompanying activity changes.

Providing such complexes were prevented from dissociating when diluted, a complex with a molecular weight greater than 1 000 000, would have been expected to separate easily from other proteins as an entity, by gel-filtration (Mowbray and Moses, 1976). Sucrose density gradient studies have also been successfully used in the isolation of large complexes (Berlyn and Giles, 1969; Gaertner and DeMoss, 1969).

Identification of multi-enzyme complexes with electron microscopy using the technique of Mowbray and Moses (1976) was also anticipated.

Finally, an attempt was to be made to obtain evidence for the channelling of intermediate metabolites within these complexes. The first method of investigation was to involve the measurement of the dilution of label from exogenously added, isotopically labelled intermediates in the end product of the multimeric system. The second was to focus on the kinetic measurements of the transient time for the formation of a steady-state within the complex (Easterby, 1981; Easterby, 1989).

PROCEDURES

List of Materials

Material	*Supplier*
chemical reagents	British Drug Houses, Poole, U.K. Fison's Apparatus, Loughborough, U.K.
cofactors	Boehringer Mannheim
coupling enzymes	Boehringer Mannheim
dye-ligand matrix	Amicon Corporation, Lexington MS, U.S.A.
enzyme substrates	Boehringer Mannheim
glass beads	Braun
iso-electric point markers	British Drug Houses
molecular weight markers	Sigma Chemical Corporation
mono Q	Pharmacia Fine Chemicals,
phenyl superose	Pharmacia Fine Chemicals,
ultrafiltration membranes	Amicon Corporation, Lexington MS, U.S.A.
yeast extract	Difco
AcA-34	Ultrogel
AcA-44	Ultrogel
DE-52	Whatman, Maidstone, U.K.
HE-7B	ICI

H4G	ICI
SBP	sedoheptulose 1,7-bisphosphate

Buffers

Buffers were made up in double distilled water, and contained sodium azide (7.7mM) and mercaptoethanol (mpte) (0.5mℓ/ℓ) (Lazaro *et al.*, 1986). To protect the enzymes against the deleterious effects of cooling, buffers contained 10% v/v glycerol (Jarabak *et al.*, 1966). Glycerol was left out of the breaking buffers to keep their buoyancy low during the ultracentrifugation of the crude extract. The buffers for hydrophobic interaction chromatography did not contain glycerol either, since it significantly increased the hydrophobicity of the buffers as well as the back pressure of the FPLC™ system. Whenever NAD was used in the purification of glyceraldehyde 3-phosphate dehydrogenase (G3PDH), 75mg was added per litre of buffer (Nicholson *et al.*, 1987). The buffer compositions were calculated using the program 'Buffer.exe' on an RM Nimbus PC network. This program takes into account the effect of the ionic strength on the thermodynamic pKa and was created by Dr. J.S. Easterby (Biochemistry Dept., Liverpool University).

Buffer Code	Nature	Ionic Strength	pH
A	Phosphate	0.985	7.5
B	Phosphate	0.025	7.5
C	Phosphate	0.050	7.5
E	Tris-HCl	0.025	8.5
F	Tris-HCl	0.392	8.5
G	Phosphate	0.221	7.0
H	Phosphate	0.392	7.7
I	Phosphate	0.028	8.0
J	Phosphate	0.130	7.5

Table II.1 The Buffers Used in the Purification.

Buffer	Enzyme to be Purified		
	G3PDH, PRK and RuBPC	Aldolase	PGK
Breaking	A	J	E
Dialysis	B	I	E
Equilibrating	C	I	E
Strong	A	H	F

Table II.2 The Combinations of Buffers Used in the Purification of any Given Enzyme.

Growth of Alga

The culture of *Scenedesmus obliquus* (Cambridge 276/6a) was based on the method of Kessler *et al.* (1957).

Autotrophic Growth

One litre flasks containing 450mℓ of medium were inoculated with *S. obliquus*. The flasks were then agitated to disperse the inoculum and the contents were transferred to glass tubes. These were placed in front of a bank of fluorescent lighting in a warm room maintained at

28°C. A mixture of 5% carbon dioxide in air was bubbled through them. After a week's growth, the cells were transferred to 15ℓ jars containing 12ℓ of medium, and allowed to grow for a further fortnight.

Heterotrophic Growth

The growth medium was supplemented with 0.5% glucose and 0.25% yeast extract (Pratt and Bishop, 1968). The alga was grown in 2ℓ conical flasks containing 1ℓ of medium, maintained at 28°C in darkened orbital incubators (Lazaro *et al.*, 1986). The alga was harvested after six days growth.

Photoheterotrophic Growth

Heterotrophic growth was also carried out in the presence of light. The alga was initially grown in 250mℓ flasks kept in illuminated orbital incubators for a week, before being transferred to 15ℓ glass jars, containing 12ℓ of heterotrophic medium, placed between two banks of white fluorescent lighting and aerated at *ca.* 4ℓ/min (Lazaro *et al.*, 1986), for a further six days in the 28°C warm room.

Harvesting of Alga

The algal cells were spun down at 2 000 × **g** for 10min. The solid green algal paste was resuspended in distilled water and then centrifuged at 2 000 × **g** for a further 10min. The green pellet was finally resuspended in a minimal volume of breaking buffer, divided into batches of about 50g wet weight of alga, and stored at -20°C (Lazaro *et al.*, 1986).

Preparation of Crude Extract

Batches of alga were thawed and 50μℓ of mercaptoethanol were added to each batch. The cells were adequately diluted with breaking buffer and were broken by grinding in the presence of 120mℓ of glass beads (diameter of 0.25-0.30mm) per batch for 10min, in a Dynomill. Efficient cooling of the breaking chamber was achieved by continuously circulating cold water, maintained at -5°C by an ice-salt mixture (Nicholson *et al.*, 1987).

The glass beads were separated from the extract by filtering the extract through a sintered glass funnel (porosity #1) and were subsequently washed with additional buffer for further recovery of broken and whole algal cells. The filtrate was centrifuged at 48 000 × **g** for 10min, the residue discarded, and the supernatant centrifuged at 190 000 × **g** for 5h. The resulting supernatant was

filtered through glass wool and then dialyzed overnight against 5ℓ of dialysis buffer (Nicholson *et al.*, 1987).

General Enzyme Purification

All protein samples were centrifuged, just before application to the chromatographic column. The phenyl superose column was pre-equilibrated with 1.2 or 1.7M ammonium sulphate in a phosphate buffer of ionic strength 0.221 and pH 7.0. All other purification columns were pre-equilibrated with the equilibrating buffer. Fractions from each chromatographic run, having activities of at least 50% of the peak fraction of the required enzyme, were pooled and concentrated.

Protein Concentration

Protein solutions were concentrated by ultrafiltration under nitrogen pressure (3.5 bar) in a stirred cell with a UM10 membrane, which restricted the passage of molecules of molecular weight higher than 10 000.

DEAE-Cellulose Chromatography

Ion exchange chromatography on DEAE-cellulose was the first step used in the purification of proteins from *S. obliquus*. Being a strong anion exchanger, the nucleic acids in the crude extract bound strongly to DEAE-cellulose. This prevented interference by nucleic acids in the estimation of proteins by measurement of the absorption of the extract at 280nm. Being the first step, DEAE-cellulose also acted as a filter, adsorbing lipids and other material which are difficult to remove from purification columns, thereby protecting the expensive FPLC™ and Ultrogel™ columns.

The preswollen, microgranular form of DEAE-cellulose (Whatman DE-52™) was prepared and recycled according to the manufacturer's instructions. The dialyzed crude extract was divided into three equal volumes and each applied to a Whatman DE-52 ion exchange column (2.2×29.5cm), previously equilibrated with equilibrating buffer. A linear salt gradient was made by mixing 220mℓ of equilibrating buffer with 220mℓ of strong buffer (Nicholson *et al.*, 1987). After eluting any unbound protein with equilibrating buffer, the salt gradient was applied to the column. Using a flow rate of approximately 50mℓ/h, 6mℓ fractions were collected and assayed for protein (E_{280}), conductivity, and the various enzyme activities.

Gel Filtration

The pooled fractions from Whatman DE-52 chromatography were concentrated down to a final volume of approximately 4mℓ (approximately 4% of the column volume), the limit set by Scopes (1975) for good resolution during gel filtration. Concentrated protein was applied to a pre-equilibrated Ultrogel™ AcA™ column (2.6 × 83.0cm) and the applied proteins were eluted with equilibrating buffer at a flow rate of 20mℓ/h. Fractions of 4mℓ were collected and assayed for the required enzyme activity. Gel filtration also provided a means of determining the molecular weight of native globular proteins (Pharmacia, 1979). Although the elution volume was a function of the Stoke's radius (Siegel and Monty, 1966), there was also a negative correlation between the elution volume and the logarithm of the molecular weight of globular proteins of a similar Stoke's radius (Andrews *et al.*, 1964).

Hydroxyapatite Chromatography

As a phosphate-containing sorbent, hydroxyapatite has been used for the separation of phosphate dependent enzymes (Life Science, 1971). The mechanism of purification is different from that of ion exchange. Proteins bind to the column through its negative (*i.e.* carboxyl and phosphate) and positive (probably calcium) groups (Bernardi, 1971).

The bulked fractions from Ultrogel AcA-34 chromatography were concentrated and applied to a hydroxyapatite column (1.6 × 27.8cm). After unbound proteins were eluted with equilibrating buffer, a linear phosphate gradient was applied, formed by mixing 500mℓ of equilibrating buffer with 500mℓ of 200mM phosphate buffer, pH 7.0, with 10% v/v glycerol (Nicholson *et al.*, 1987). The flow rate was 20mℓ/h, and six fractions (8mℓ) of highest enzyme activity were pooled.

Triazine Dye-ligand Chromatography

Various triazine dyes, obtained from ICI, had previously been covalently bound to an Amicon matrix. These were made into columns of 2mℓ for analytical studies. For preparative uses, columns of 15mℓ were used. Protein samples were applied to a dye column, and the equilibrating buffer was then used to elute the unbound proteins. Bound proteins were eluted with either strong buffer or with the equilibrating buffer containing 10mM ATP or 12mM 3-phosphoglycerate (PGA), to elute phosphoglycerate kinase (PGK), and 0.56mM fructose 1,6-bisphosphate (FBP), to elute FBP aldolase.

Fast Protein Liquid Chromatography

A Pharmacia FPLC system was used for ion exchange and hydrophobic interaction chromatography according to the manufacturer's instructions.

Ion Exchange Chromatography

Samples were applied to either an HR 5/5 or HR 10/10 mono Q™ column and eluted using either a phosphate gradient (buffers C and A) or a Tris-HCl gradient (buffers E and F).

Hydrophobic Interaction Chromatography

In the presence of a high concentration of ammonium sulphate, the exposed hydrophobic regions on proteins adsorbed onto the hydrophobic phenyl residues bound to the superose base-matrix. Selective elution was effected by a gradual decrease in hydrophilicity brought about by a decreasing concentration of ammonium sulphate (Pharmacia, 1986).

Samples were applied to an HR 5/5 phenyl superose column and eluted using a reverse ammonium sulphate gradient (buffer G and 1.2 or 1.7M ammonium sulfate in buffer G).

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed in a discontinuous buffer system in the vertical plane, according to the method of Laemmli (1970). The gels were stained with Coomassie blue R250, and subsequently destained with a 30% methanol and 10% acetic acid solution. The molecular weight of the subunit of a protein under investigation was determined by comparing its electrophoretic mobility with those of protein subunits of known molecular weight. The mobility was inversely related to the logarithm of the molecular weight of the subunit (Pharmacia, 1980).

Iso-Electric Focusing

Aldolase samples were focused according to a method devised by Giulian *et al.* (1984). The presence of aldolase was detected by the use of an enzyme activity stain or by western blotting.

The activity stain was developed using a modification of the method of Susor *et al.* (1975). The gel was incubated at 37°C in the dark in 25mℓ of a solution containing the components of the NADH detection system (0.58mg phenazine methosulphate and 10.00mg nitro blue

tetrazolium) and the aldolase assay (17.50mg NAD, 495.0μmoles PGA, 65μmoles ATP and 20U NADH-G3PDH).

The iso-electric point of aldolase was estimated by iso-electric focusing. A set of coloured marker proteins of known iso-electric points was focused together with aldolase. Soon after blotting, the markers were clearly visible on the blot. Before they disappeared, their positions were marked on the blot with a pencil. The pI of aldolase was obtained from a plot of the distance travelled by a focused protein versus its pI (Pharmacia, 1982).

Raising of Antisera

An emulsion of the purified G3PDH, PRK or RuBPC was made with an equal volume of complete Freund's adjuvant. The emulsified protein (*ca.* 500μg/4-5mℓ) was injected intramuscularly into the back of a New Zealand rabbit. A booster was given subcutaneously, five weeks later in incomplete Freund's adjuvant (Klein *et al.*, 1983). Approximately, 7-9mℓ of blood was obtained four to five weeks after every dose. It was left at room temperature for approximatrely 4h, to give it adequate time to clot. The serum was frozen in 25μℓ batches.

The antisera to phosphoglycerate kinase (PGK) and aldolase were raised in Dutch rabbits. Since these rabbits were of a smaller size, a lower amount of protein was required to immunise them. About

150μg of PGK or aldolase was used for each dose. The time intervals observed were the same as for the previous antigens, but no more than 2-3mℓ of blood was obtained from a single bleed.

Western Blotting

SDS-PAGE or iso-electrically focused gels were blotted on to nitrocellulose (0.45μm pore size) using an LKB Transphor electroblotter according to the manufacturer's instructions. The blots were probed using the following method (S. Clasper, personal communication):

All steps were performed at room temperature in 50mℓ of phosphate buffered saline (10mM NaH_2PO_4, 150mM NaCl, pH 7.5) plus 0.2% (v/v) Tween 20 on a shaker except where stated.

1. The blot was incubated with 25μℓ of rabbit antiserum for two hours.
2. The blot was washed three times for five minutes each.
3. 20μℓ of horse radish peroxidase conjugated goat anti-rabbit IgG was added and the blot incubated for two hours.
4. As in '2.'
5. 10mg of 3,4,3'4'-Tetra-aminobiphenyl hydrochloride and 100μℓ of hydrogen peroxide were added.
6. When the optimum colour intensity was achieved the blot was washed thoroughly.

7. The blot was immersed in an aqueous solution of 0.9% sodium chloride containing 0.5% copper sulphate for a few minutes to intensify the colour before being washed in water and dried.

Protein Estimation

The protein elution profile for Whatman DE-52 chromatography was obtained either by measuring the absorption at 280nm of each fraction, or by continuously monitoring the eluant at 280nm.

The tannin precipitation method of Mejbaum-Katzenellenbogen and Dobryszycka (1959) was used for determining protein concentrations used in calculating specific activities and the protein content of pure enzyme preparations, prior to raising antibodies

Protease Assay

The protease activity in crude extracts was measured by the rate of hydrolysis of azocasein (Beynon, 1989).

Estimation of Cytochrome c

S. obliquus cytochrome c does not bind to Whatman DE-52 under the conditions applied. An oxidised versus reduced difference spectrum was obtained by using the basic protein eluted from Whatman DE-52. Oxidation and reduction were carried out by the addition of potassium ferricyanide and sodium dithionite, respectively (Henderson and Rawlinson, 1956).

Enzyme Assays

The activities of all enzymes were measured by following the decrease in absorbance at 340nm (Racker, 1947), at 30°C in a volume of 1mℓ in a plastic cuvette. All solutions were made up in a Tris-HCl buffer of ionic strength 0.038 and pH 7.5. One unit of enzyme activity was defined as the amount of enzyme required to catalyse the oxidation of one micromole of substrate per minute (Woodrow *et al.*, 1979), which was equivalent to the oxidation of one micromole of NADH per minute, under the above conditions. The only exception occurred in the case of the aldolase assay. Here, the rate of NADH consumption was twice that of the substrate (Krüger and Schnarrenberger, 1983). The amount of NADH oxidised, was calculated using the molar absorbance of NADH as 6.22×10^3 at 340nm (Jagannathan *et al.*, 1956).

Glyceraldehyde 3-Phosphate Dehydrogenase

The assay for NADH-G3PDH consisted of 2.60μmol. ATP, 0.21μmol. NADH, 11.3U PGK, 19.80μmol. PGA, 3.17μmol. cysteine-hydrogen chloride, 10.00μmol. $MgCl_2$ and 50.10μmol. KCl.

For NADPH-G3PDH, the assay for NADH-G3PDH assay was used, except that 0.30μmol. NADPH was substituted for 0.21μmol. NADH.

Phosphoribulokinase

The assay was based on the dependence of the dephosphorylation of phosphoenolpyruvate (PEP) by pyruvate kinase (PK) (EC 2.7.1.40) on ADP. Formation of pyruvate, the product of this reaction, was determined with lactate dehydrogenase (LDH) (EC 1.1.1.27) (Racker, 1957). The assay consisted of 2.00μmol. ribose 5-phosphate (R5P), 2.70μmol. phosphoenolpyruvate, 2.60μmol. ATP, 0.21μmol. NADH and 3.8U phosphoriboisomerase (EC 5.3.1.6), 5.0U PK, 5.00μmol. reduced glutathione and 13.8U LDH, 10.00μmol. $MgCl_2$ and 50.10μmol. KCl.

Ribulose 1,5-Bisphosphate Carboxylase

The transformation of R5P to ribulose 1,5-bisphosphate and then to PGA, was measured by the reduction of the latter by NADH in the presence of ATP, PGK and NADH-G3PDH (EC 1.2.1.12) (Racker, 1957). 30μℓ of enzyme preparation was activated by addition of 29.76μmol.

sodium hydrogencarbonate and 61.48μmol. magnesium chloride in a total volume of 90μℓ of a Tris-HCl buffer of ionic strength 0.038 and pH 7.5. The assay consisted of 3.8U PRI, 2.00μmol. R5P, _._U PRK, 0.21μmol. NADH, 0.97μmol. dithiothreitol (DTT), 2.60μmol. ATP, 0.8U NADH-G3PDH, 11.3U PGK, 10.12μmol. sodium hydrogencarbonate, 7.0U creatine kinase (EC 2.7.3.2) and 9.80μmol. creatine phosphate., 10.00μmol. $MgCl_2$ and 50.10μmol. KCl.

Aldolase

The assay was a modification of that devised by (Wu and Racker, 1959) and consisted of 0.21μmol. NADH, 0.97μmol. DTT, 2.00μmol. fructose 1,6-bisphosphate, 8.5U α-glycerophosphate dehydrogenase and 10.0U triosephosphate isomerase.

Phosphoglycerate Kinase

The PGK reaction was coupled to the G3PDH reaction (Scopes, 1975). The assay consisted of 0.21μmol. NADH, 19.80μmol. PGA, 2.60μmol. ATP, 0.97μmol. DTT and 0.8U NADH-G3PDH, 10.00μmol. $MgCl_2$ and 50.10μmol. KCl.

GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE, PHOSPHORIBULOKINASE AND RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE

Prior to the commencement of the project, much research had been carried out on the glyceraldehyde 3-phosphate dehydrogenase-phosphoribulokinase (G3PDH-PRK) complex in this laboratory. Consequently, the first enzymes to be purified in the project were G3PDH and PRK. Since ribulose 1,5-bisphosphate carboxylase (RuBPC) co-eluted with PRK during ion exchange chromatography on Whatman DE-52™, RuBPC was the third enzyme to be purified.

Heterotrophically grown *Scenedesmus obliquus* was used as the source of G3PDH, PRK and RuBPC because the cellular yield of heterotrophically grown alga was approximately four fold more than that obtained through autotrophic growth (Eppley and Macias, 1962).

First Purification Step

The extracted soluble proteins were applied to three similar Whatman DE-52 ion exchange columns (Fig. III.1). The first peak of eluted protein (fractions 1-11) consisted of basic protein which did not bind to the anion exchanger at pH 7.5. The second protein peak included phosphoglycerate kinase and fructose 1,6-bisphosphate aldolase, which were subsequently purified.

Since the alga was grown heterotrophically, the chloroplast G3PDH present in the crude extract, showed NADH-dependent activity and was complexed with PRK (O'Brien *et al.*, 1976; Nicholson *et al.*, 1987). In the column eluant, NADPH-dependent activity accounted for less than 25% of the total eluted G3PDH activity and this NADPH-dependent activity eluted from the column, along with the NADH-dependent activity. Such NADPH-dependent activity was due to the large G3PDH-PRK complex. Ion exchange chromatography caused the G3PDH-PRK complex to dissociate into a form of G3PDH with NADPH-dependent activity and an active form of PRK. This dissociation could be prevented by the addition of NAD to the buffer system (Powls and Woodrow, 1981; Lazaro *et al.*, 1986). NAD is known to promote subunit interactions in G3PDH (Seydoux *et al.*, 1973).

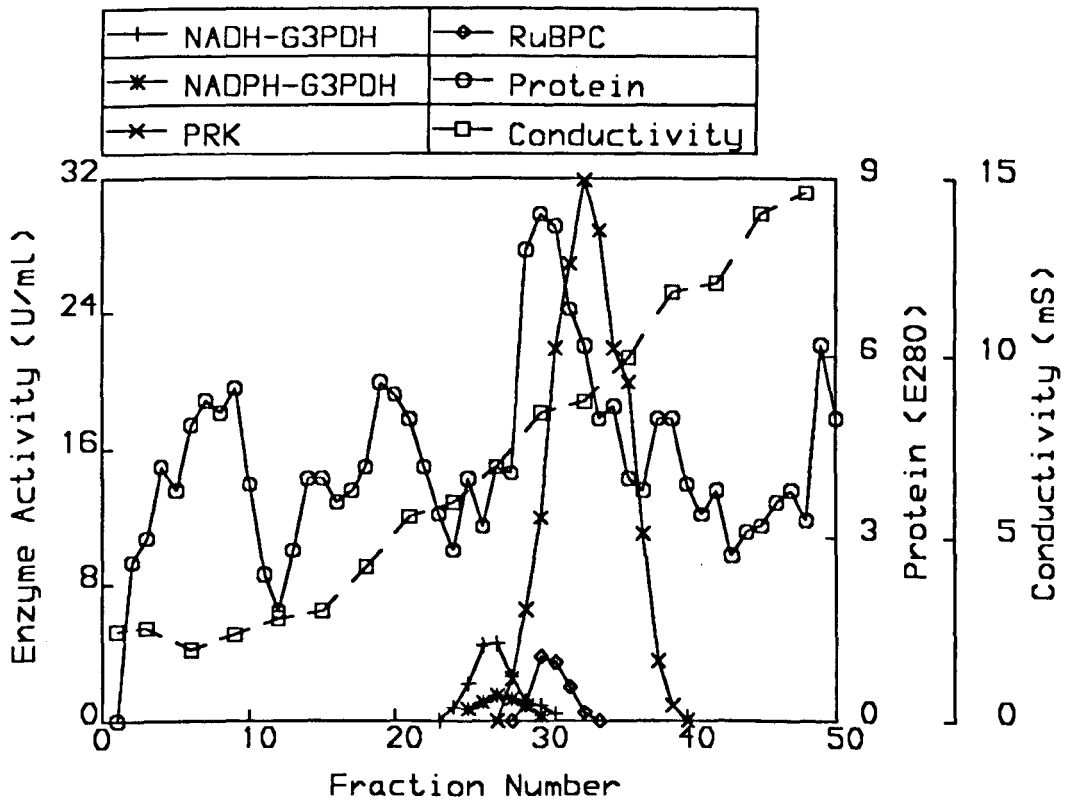


Figure III.1 Ion exchange of crude extract from heterotrophically grown *S. obliquus* on Whatman DE-52.

The gradient was developed by mixing phosphate buffers of ionic strength 0.050 and 0.985 on a 2.2 × 29.5cm column. Elution was performed in the presence of NAD at pH 7.5 with a flow rate of 50mℓ/h and 6mℓ fractions were collected. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

In heterotrophically grown *S. obliquus*, not all the PRK was latent and complexed with G3PDH. A substantial proportion of PRK was free and hence active. This active PRK was eluted in fractions 30-40.

When the aim was to purify PRK, NAD was not added to the buffers. Consequently the G3PDH-PRK complex dissociated to give the form of G3PDH showing NADPH-dependent activity as well as active PRK. This newly formed PRK eluted from the column in the same fractions as did the original active PRK.

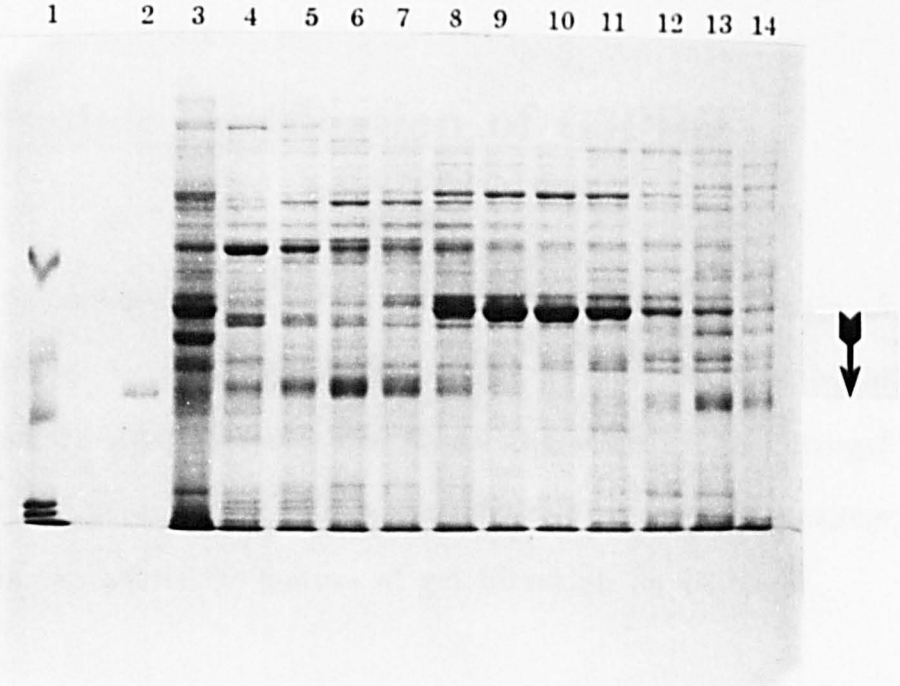
The elution profile was similar to that seen, when the buffers contained NAD (Fig. III.1), except that in the absence of NAD, there was much more of both the active form of PRK and NADPH dependent G3PDH. A corresponding decrease in the NADH-dependent G3PDH activity was observed. RuBPC was eluted in the fractions containing the major protein in the extract, and its activity was unaffected by NAD.

The spectrophotometric assay for RuBPC, involving the oxidation of NADH, was time consuming as it required a preliminary activation step. The assay was also characterised by an unusually long hysteresis. Thus, for routine purification, it was easier to detect the presence of RuBPC in the various fractions by SDS-polyacrylamide gel electrophoresis (PAGE). The larger subunit of RuBPC was clearly visible in the SDS-PAGE of the crude extract, and was the major protein in fractions 30-32 (Fig. III.2). This was not surprising, as RuBPC constitutes about 65% of the total protein in the aqueous

Figure III.2 SDS-PAGE of fractions eluted from Whatman DE-52.

[1] M_r marker proteins (66 000, 45 000, 36 000 and 29 000); [3] crude extract from heterotrophically grown *S. obliquus*; [4-14] fractions 25-33, 35 and 37 eluted from Whatman DE-52 chromatography (Fig. III.1).

G3PDH, PRK and RuBPC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extracts of higher plants (see review by Foyer (1948)). There was very little resolution of peptides below a molecular weight of 29 000, probably because the gel may have been overloaded with protein. Consequently, the smaller subunit of RuBPC, having a molecular weight of less than 29 000, was not visible.

Further Purification of G3PDH

The best fractions containing G3PDH activity from the three Whatman DE-52 columns were pooled, concentrated and divided into two equal portions. Each portion was subjected to gel filtration on an Ultrogel AcA-34™ column (Fig. III.3). The considerable spread in enzyme activity was a characteristic feature of gel filtration on Ultrogel.

The advantage in keeping G3PDH in the form with NADH-dependent activity was quite evident. Due to its high molecular weight, the G3PDH-PRK complex eluted much earlier than the majority of other proteins. Gel filtration in the absence of NAD, did not cause the depolymerization of G3PDH, probably because it was a much gentler technique than ion exchange chromatography. Hence, it was not necessary to add NAD to the buffers used for gel filtration of G3PDH.

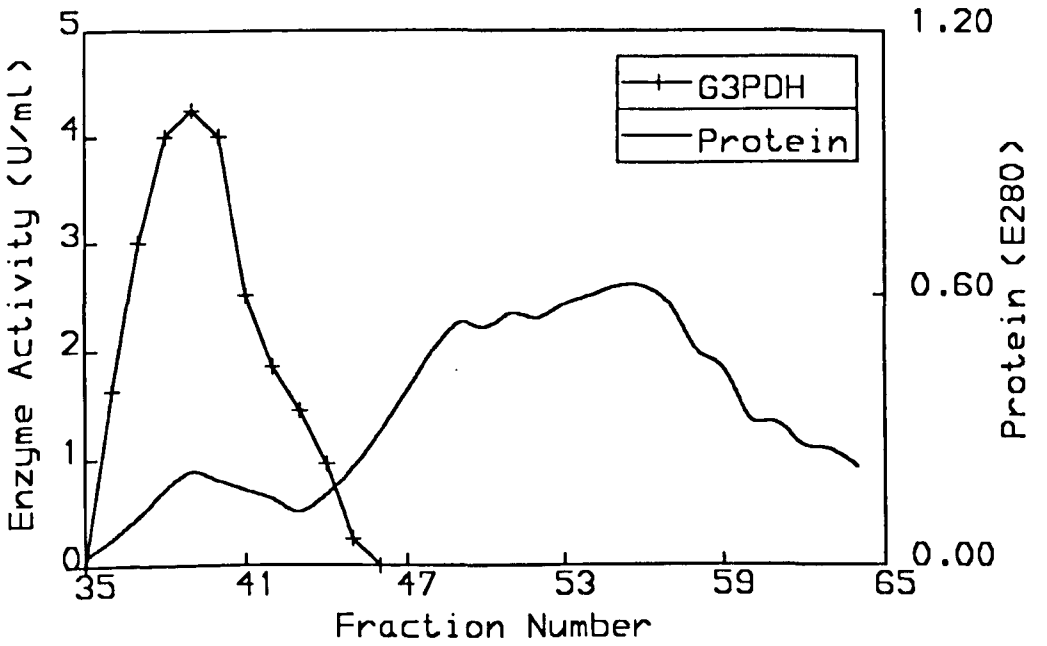


Figure III.3 Gel filtration of NADH-G3PDH (G3PDH-PRK complex) on Ultrogel AcA-34.

The extract from heterotrophically grown *S. obliquus* had previously been purified on Whatman DE-52. Elution was performed in the absence of NAD on a 2.6 × 83.0cm column at pH 7.5 with a flow rate of 20mℓ/h. Fractions of 3mℓ were collected. The eluting buffer was phosphate with an ionic strength of 0.050 and supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

FPLC on Mono-Q

In the Absence of NAD

The fractions with the highest NADH-dependent G3PDH activity from gel filtration were pooled, divided into three portions and chromatographed on an FPLC mono Q™ anion exchange column in the absence of NAD. This promoted depolymerization of the complex and the consequent separation of the NADPH-dependent form of G3PDH and active PRK (Fig. III.4). A small amount of NADH-dependent G3PDH activity was associated with the depolymerised form of the enzyme which had high NADH-dependent activity. The fractions containing the highest activities of G3PDH were pooled and frozen.

During this depolymerization, PRK was not eluted as a distinct peak. Moreover, the eluted PRK activity was very much less than the NADPH-dependent G3PDH (Fig. III.4). This contrasts with the situation when depolymerisation of the complex was promoted by incubation with DTT and NADPH. In the latter case, although both PRK and NADPH-dependent activities were stimulated (Fig. III.5), the resulting PRK activity was more than double the resulting NADPH-dependent G3PDH.

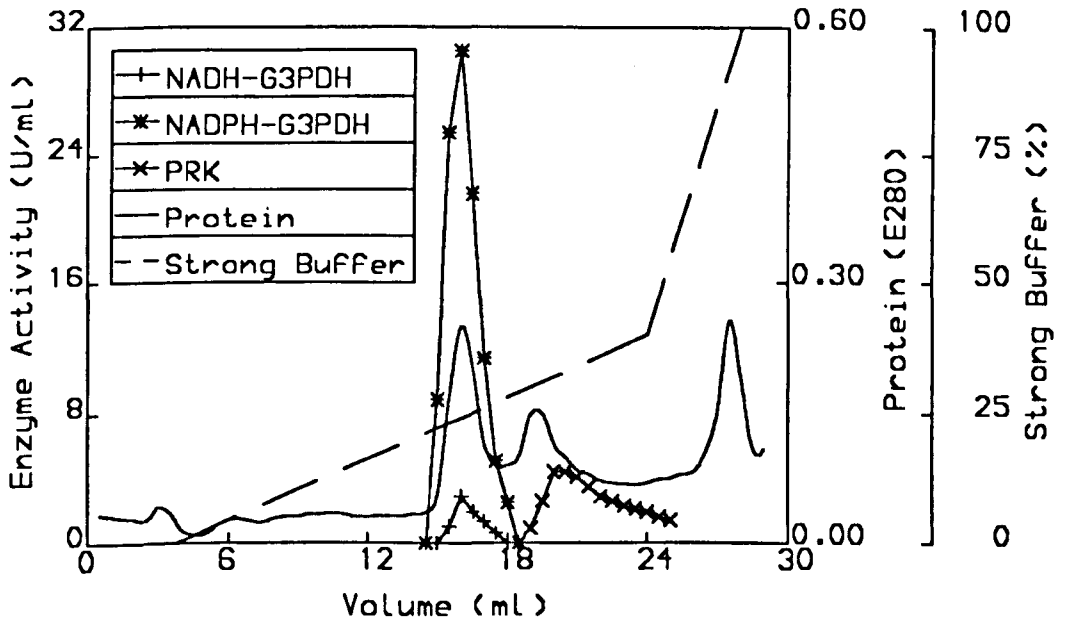


Figure III.4 Ion exchange chromatography of NADH-G3PDH (G3PDH-PRK complex) on FPLC mono Q.

The gradient was developed by mixing phosphate buffers of ionic strength 0.050 and 0.985 on a HR5/5 column. Elution was performed in the absence of NAD at pH 7.5 with a flow rate of 1mℓ/min. The extract from heterotrophically grown *S. obliquus* had previously been eluted from Whatman DE-52 and Ultrogel AcA-34. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

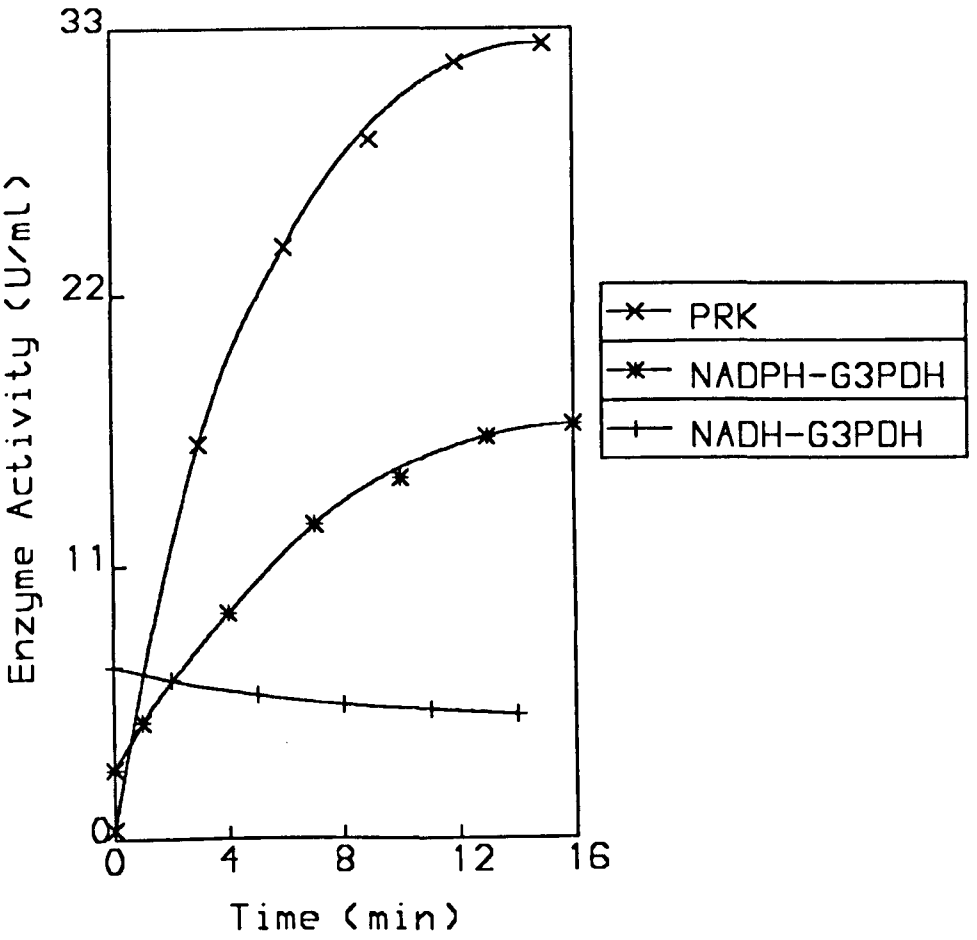


Figure III.5 Effect of incubating NADH-G3PDH (G3PDH-PRK complex) with DTT and NADPH.

50μℓ of NADH-G3PDH (G3PDH-PRK complex) previously eluted from Whatman DE-52 and Ultrogel AcA-34 was incubated with 1μmol. NADPH and 1μmol. DTT. The incubation volume was made up to 200μℓ with Tris-HCl buffer of ionic strength 0.038 and pH 7.5.

In the Presence of NAD

If the FPLC on mono Q was carried out in the presence of NAD, the G3PDH-PRK complex was partially stabilised, resulting in the elution of two major peaks of G3PDH activity (Fig. III.6). The first showed predominantly NADPH-dependent activity and was due to dissociated G3PDH, whilst the second was due to the complex and was characterised by NADH-dependent activity and a small amount of NADPH-dependent activity. NAD absorbs strongly at 280nm and this interfered with the protein estimation. The best fractions of the latter form were pooled and frozen.

Gel Filtration of PRK and RuBPC

Fractions eluted from the Whatman DE-52 columns containing both RuBPC and PRK were concentrated and applied in two batches to the Ultrogel AcA-34 gel filtration column. RuBPC, being the larger molecule, eluted before PRK (Fig. III.7). Although the two enzymes co-eluted on the Whatman DE-52 column, if this had been due to molecular interaction it was not apparent during gel filtration. Since gel filtration was believed to be a gentler technique, it was more likely that a PRK-RuBPC complex, if present, would have dissociated during ion exchange chromatography, rather than during gel filtration.

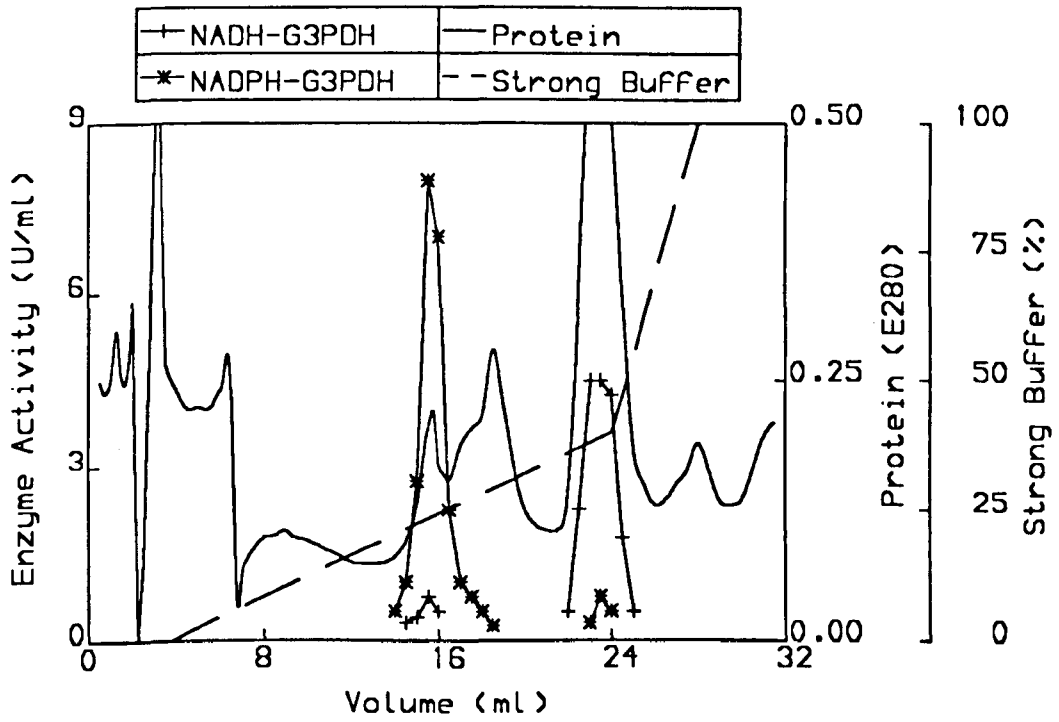


Figure III.6 Ion exchange chromatography of NADH-G3PDH (G3PDH-PRK complex) on FPLC mono Q.

The gradient was developed by mixing phosphate buffers of ionic strength 0.050 and 0.985 on a HR5/5 column. Elution was performed in the presence of NAD at pH 7.5 with a flow rate of 1mℓ/min. The extract from heterotrophically grown *S. obliquus* was previously purified on Whatman DE-52 and Ultrogel AcA-34. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

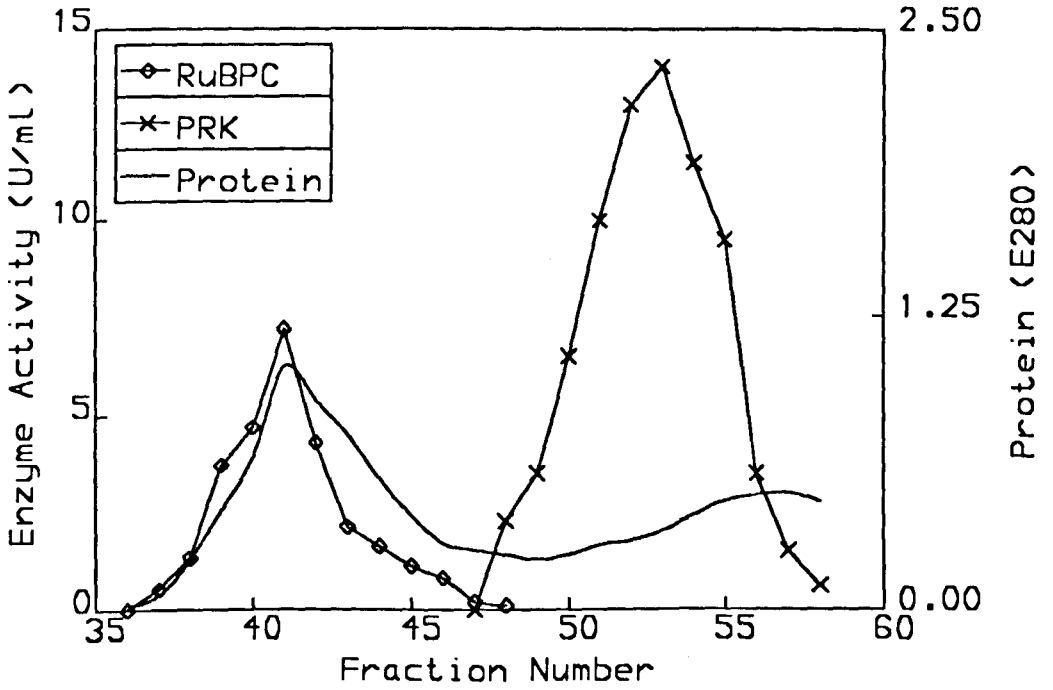


Figure III.7 Gel filtration of RuBPC and PRK on Ultrogel AcA-34.

Elution was performed in the absence of NAD on a 2.6 × 83.0cm column at pH 7.5 with a flow rate of 20mℓ/h. The extract from heterotrophically grown *S. obliquus* had previously been purified on Whatman DE-52. The eluting buffer was phosphate with an ionic strength of 0.050 and supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol. Fractions of 3mℓ were collected.

SDS-PAGE of fractions eluted from gel filtration indicated that the peak fraction of RuBPC was almost pure, whilst there were many bands present even in the best PRK fraction (Fig. III.8). After staining with Coomassie blue, the larger subunit of RuBPC was more prominent than the smaller subunit. The best fractions of both enzymes were pooled separately and concentrated.

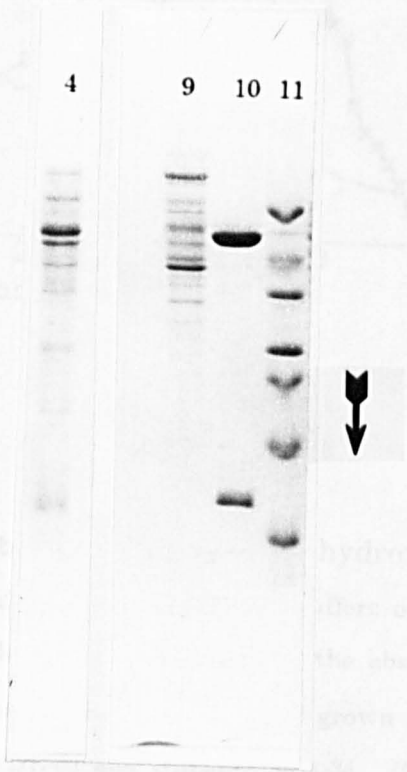
Further Purification of PRK

The PRK preparation was further purified by chromatography on a hydroxyapatite column using a linear gradient of phosphate (Fig. III.9). There was a considerable spread in enzyme activity, which may have been partly caused by overloading the column.

The fractions eluted from hydroxyapatite showing the highest PRK activity were subjected to ion exchange chromatography on the FPLC mono Q column (Fig. III.10). The single major protein peak was eluted in the same fractions as those with PRK activity. The fractions with the highest PRK activity (fractions 13-15) were pooled and their purity checked by SDS-PAGE.

Figure III.8 SDS-PAGE of RuBPC and PRK eluted from Ultrogel AcA-34.

[4] crude extract from heterotrophically grown *S. obliquus*; [9] peak fraction of PRK eluted from Ultrogel AcA-34 (Fig. III.7); [10] peak fraction of RuBPC eluted from Ultrogel AcA-34 (Fig. III.7) and [11] M_r marker proteins (66 000, 45 000, 36 000, 29 000, 24 000, 20 000 and 14 200).



G3PDH, PRK and RuBPC

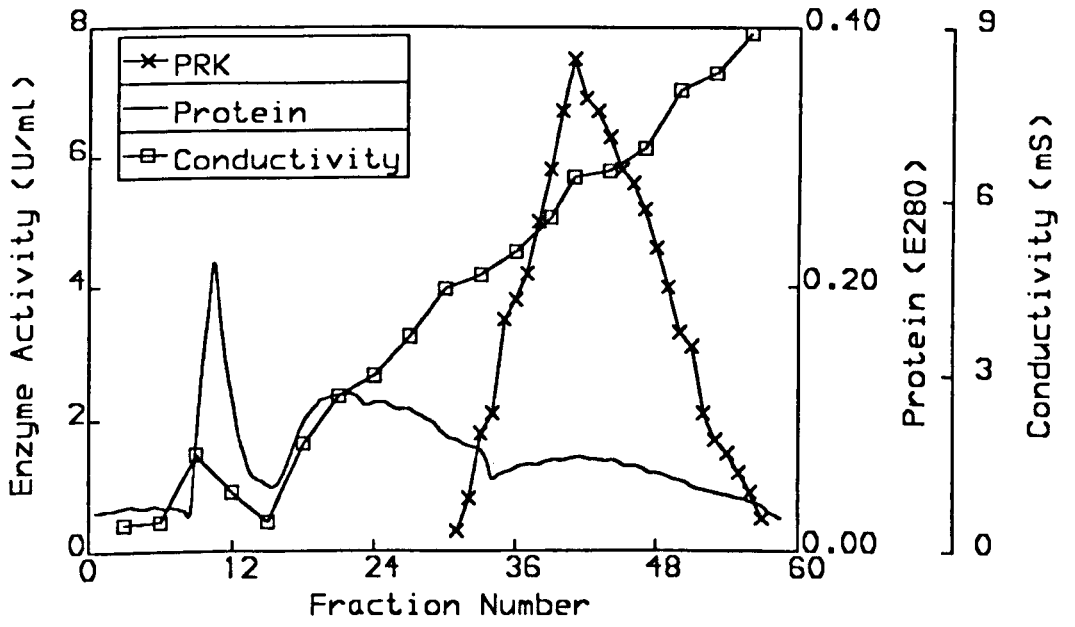


Figure III.9 Chromatography of PRK on hydroxyapatite.

The gradient was developed by mixing phosphate buffers of ionic strength 0.050 (pH 7.5) and 0.450 (pH 7.0). Elution was performed in the absence of NAD with a flow rate of 20mℓ/h. The extract from heterotrophically grown *S. obliquus* had previously been purified on Whatman DE-52 and Ultrogel AcA-34. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol. Fractions of 8mℓ were collected.

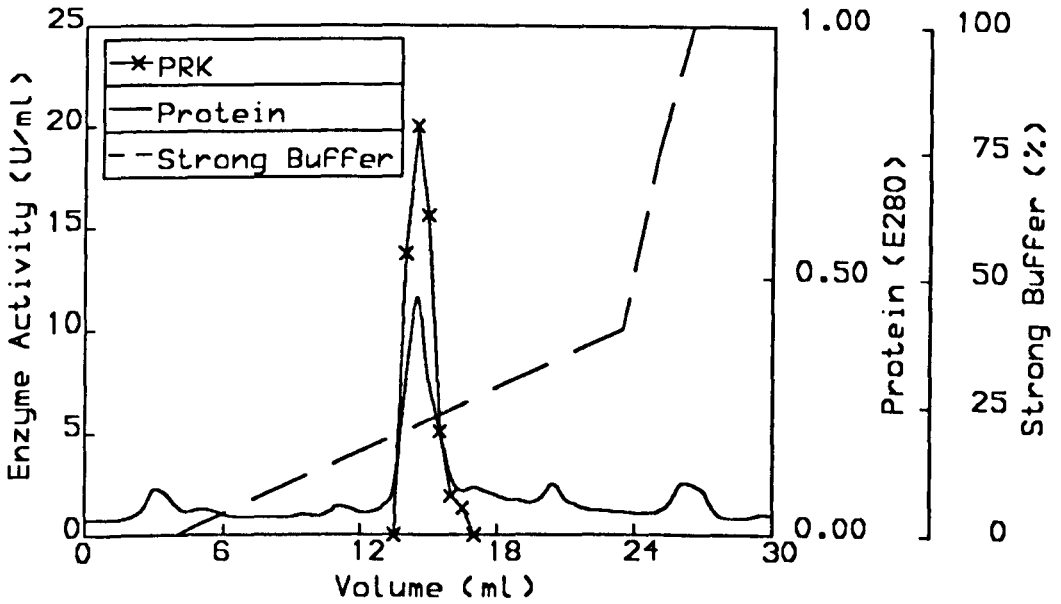


Figure III.10 Ion exchange chromatography of PRK on FPLC mono Q.

The gradient was developed by mixing phosphate buffers of ionic strength 0.050 and 0.985 on a HR5/5 column. Elution was performed in the absence of NAD at pH 7.5 with a flow rate of 1mℓ/min. The extract from heterotrophically grown *S. obliquus* had previously been purified on Whatman DE-52, Ultrogel AcA-34 and by hydroxyapatite chromatography. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

SDS-PAGE of G3PDH and PRK

SDS-PAGE of the G3PDH-PRK complex, NADPH-dependent G3PDH and PRK indicated that the three enzyme preparations had been completely purified (Fig. III.11). PRK and NADPH-dependent G3PDH were each homopolymers, whilst the complex was composed of subunits of two different sizes, as shown earlier (Powls *et al.*, 1988). The molecular weights estimated, were 42 000 for PRK, 39 000 for NADPH-dependent G3PDH and 39 000 and 42 000 for the G3PDH-PRK complex (Fig. III.12).

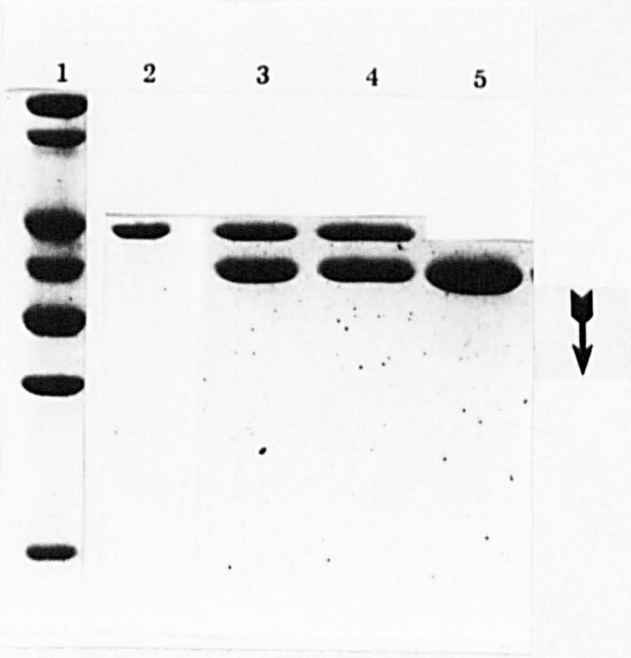
Further Purification of RuBPC

The best fractions of RuBPC from gel filtration were chromatographed on FPLC mono Q (Fig. III.13). There was only one significant protein peak, which co-eluted in fractions possessing RuBPC activity.

SDS-PAGE of the RuBPC eluted from FPLC mono Q indicated that it was completely pure (Fig. III.14). As expected, it was composed of subunits of two different sizes. RuBPC from various other plant species, such as *Spinacia oleracea* leaf (Sugiyama and Akazawa, 1970) and the marine alga, *Porphyra umbilicalis* (Hill *et al.*, 1988) was also composed of subunits of two different sizes. The molecular weights of the subunits were 15 000 and 51 000 (Fig. III.15), which was

Figure III.11 SDS-PAGE of purified NADPH-G3PDH, G3PDH-PRK complex and PRK.

The extracts from heterotrophically grown *S. obliquus* had been purified on Whatman DE-52, Ultrogel AcA-34 and FPLC mono Q. The PRK preparation was also subjected to hydroxyapatite chromatography. [1] M_r marker proteins (67 000, 57 000, 43 000, 40 000, 35 000, 31 000 and 21 000); [2] purified PRK; [3] purified G3PDH-PRK complex; [5] purified NADPH-G3PDH.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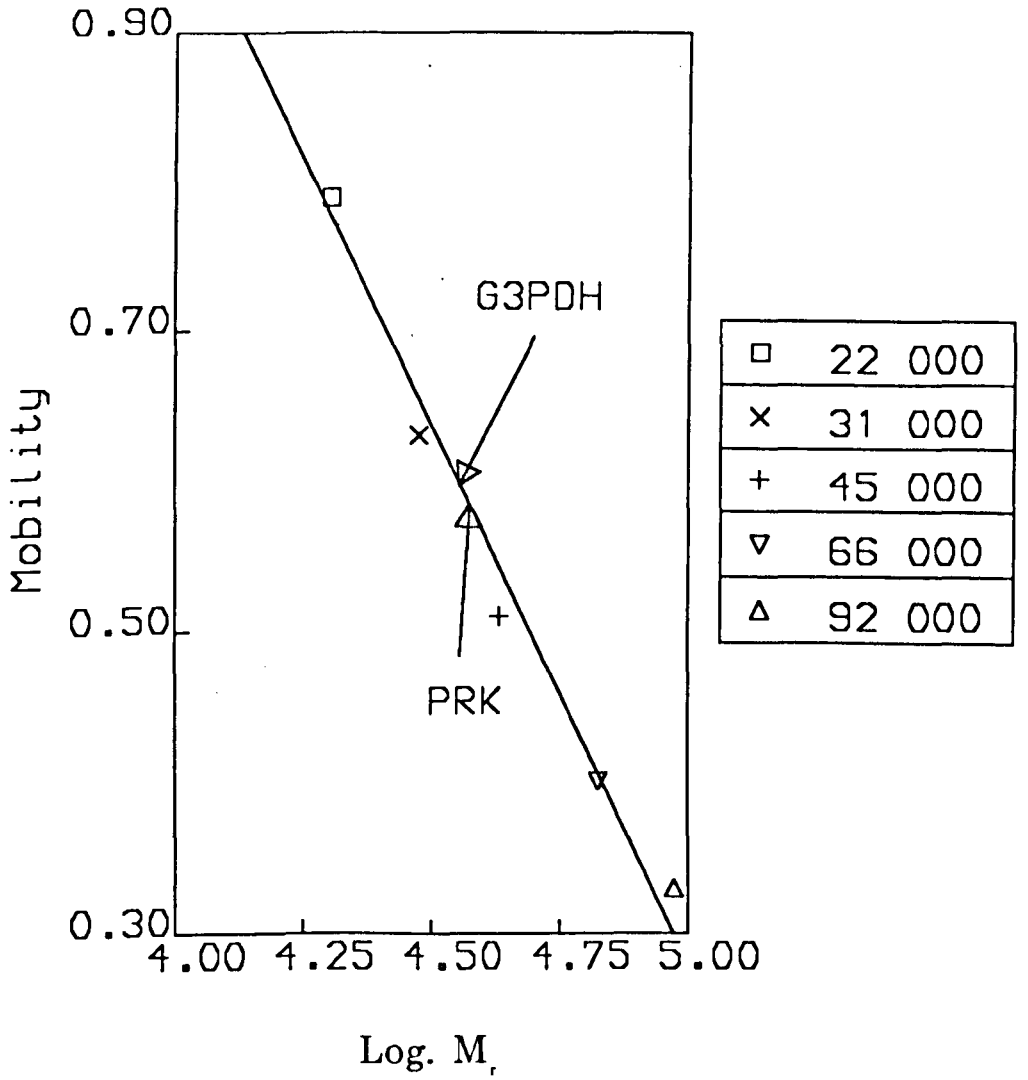


Figure III.12 M_r determination of the subunits of PRK and G3PDH by SDS-PAGE.

The relative mobilities were measured from the gel and the above plot was used to obtain their M_rs.

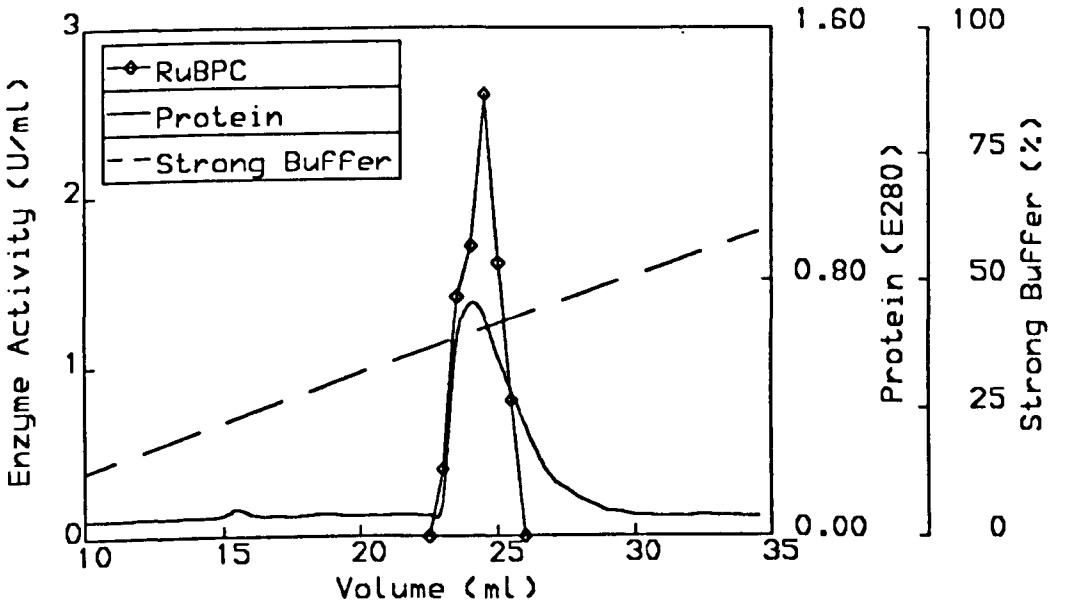
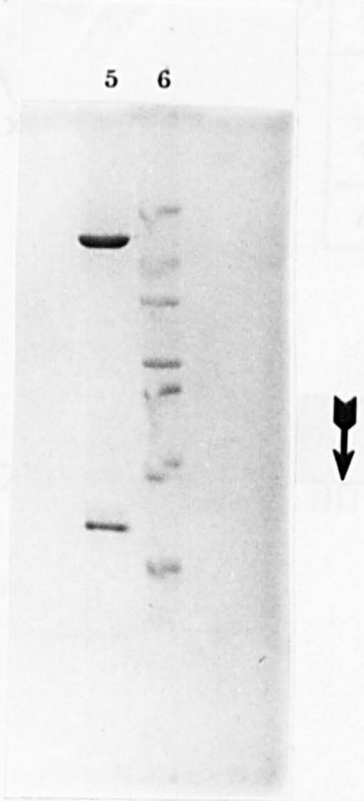


Figure III.13 Ion exchange chromatography of RuBPC on FPLC mono Q.

The gradient was developed by mixing phosphate buffers of ionic strength 0.050 and 0.985 on a HR5/5 column. Elution was performed in the absence of NAD at pH 7.5 with a flow rate of 1mℓ/min. The extract from heterotrophically grown *S. obliquus* had previously been purified on Whatman DE-52 and Ultrogel AcA-34. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

Figure III.14 SDS-PAGE of purified RuBPC.

The extract from heterotrophically grown *S. obliquus* was purified on Whatman DE-52, Ultrogel AcA-34 and FPLC mono Q. [5] purified RuBPC; [6] M_r marker proteins (66 000, 45 000, 36 000, 29 000, 24 000, 20 000 and 14 200).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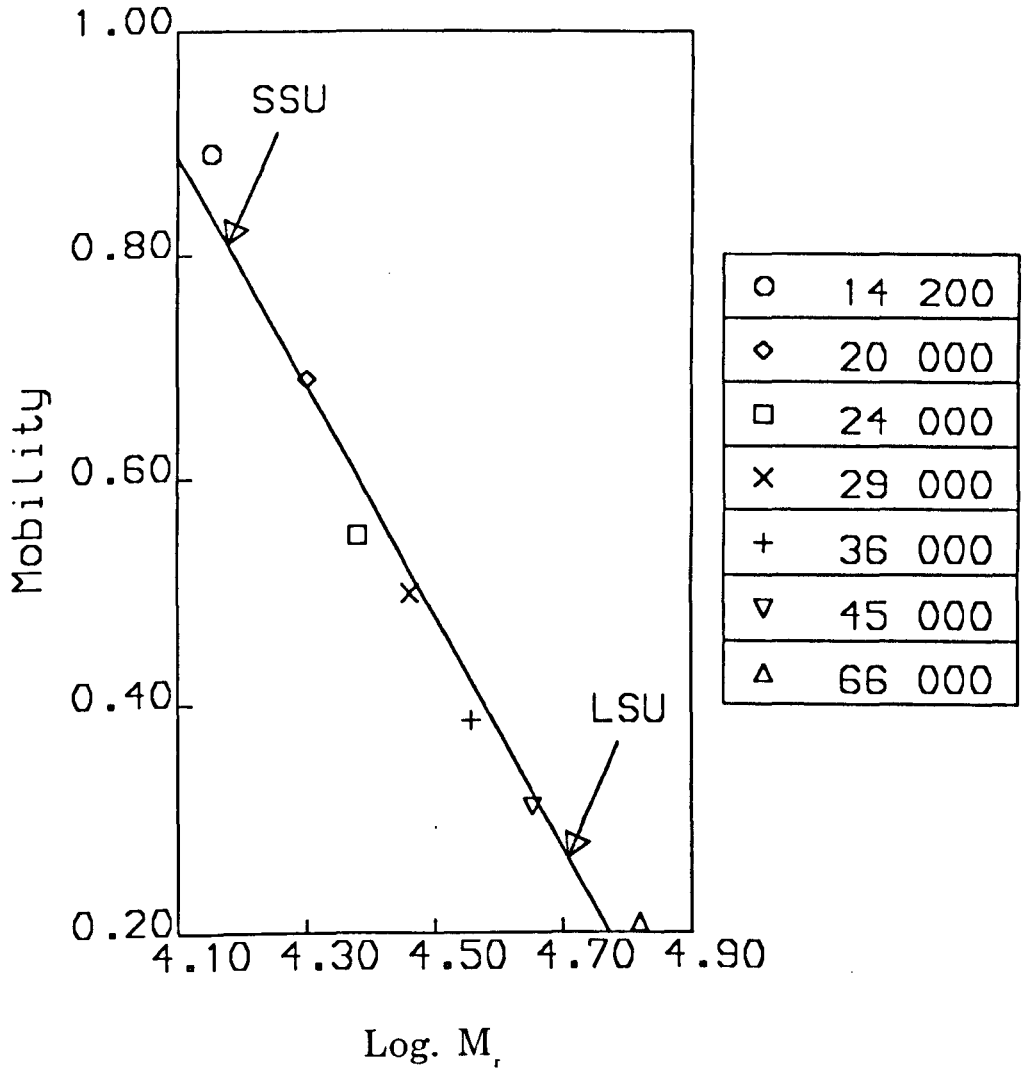


Figure III.15 M_r determination of RuBPC subunits by SDS-PAGE.

The relative mobilities were measured from the gel and the above plot was used to obtain their M_rs.

comparable to the values of 17 000 and 51 500, obtained for the RuBPC subunits obtained from *P. umbilicalis*.

Antisera

Rabbits were used to raise antisera against all three purified enzymes.

Western blots of SDS-PAGE of the purified enzymes were used to detect whether antibodies had been raised, whilst blots of SDS-PAGE of the crude extract of *S. obliquus* were used to test the specificity of the antibodies for their corresponding antigens.

The antibodies raised against G3PDH, PRK and RuBPC were stored at -20°C, whilst other key enzymes of the RPPP were purified, and antisera raised against them.

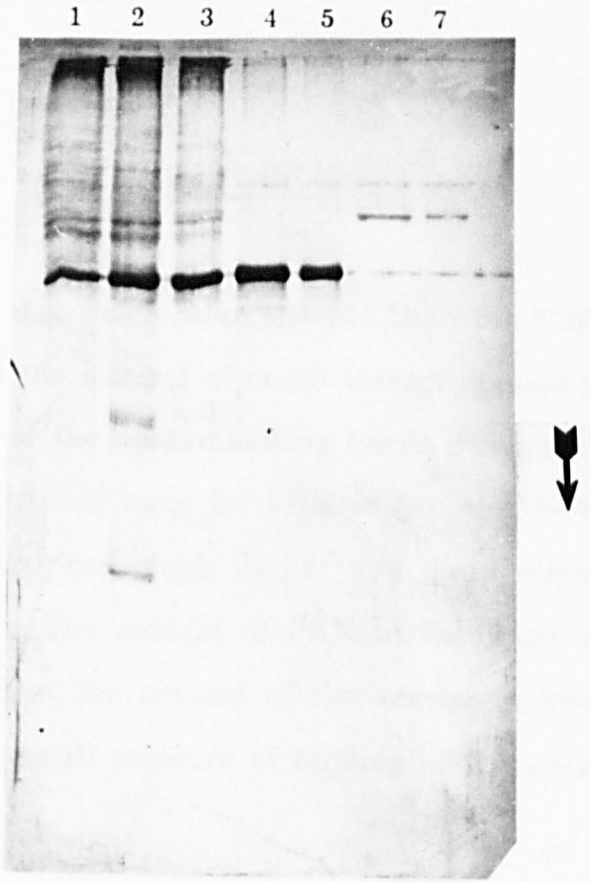
Anti-G3PDH

The antibody to G3PDH not only bound to the G3PDH subunit, but also to several other peptides, predominantly of higher molecular weight than G3PDH in the crude extract (Fig. III.16).

There was a slight amount of cross-reaction with the PRK subunit, a constituent of the G3PDH-PRK complex from which the G3PDH

Figure III.16 Western blot using antiserum raised against G3PDH.

The protein samples were subjected to SDS-PAGE prior to blotting. [1-3] samples of crude extract from heterotrophically grown *S. obliquus*; [4 and 5] samples of NADH-G3PDH; [6 and 7] samples of purified PRK.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subunit, used to raise the antibody, had been isolated. The other bands giving a positive reaction in the blot were probably an artefact of blotting experiment, as they were observed in the tracks containing the purified G3PDH and PRK enzymes. The specificity of this antibody preparation was unfortunately not high enough to be used in a search for multi-enzyme complexes.

Anti-PRK

The PRK antibody was much more specific than the G3PDH antibody (Fig. III.17). When the amount of crude extract applied to SDS-PAGE was reduced, most of the contaminating bands disappeared, leaving a distinct and easily visible band for PRK in the blot. The PRK band in the crude extract was much darker and more distinct than the purified PRK band. The amount of PRK in the crude extract track was much higher than the amount of the enzyme in the PRK track. The blot detected a small measure of binding of the antibody to both subunits of RuBPC.

Anti-RuBPC

The western blot of the antisera raised against RuBPC (Fig. III.18) showed it to be the best of the antibody preparations. There was no hint of a cross-reaction with the PRK subunit. As in the case of the blot against PRK, the amount of RuBPC applied to the SDS-PAGE

Figure III.17 Western blot using antiserum raised against PRK.

The protein samples were subjected to SDS-PAGE prior to blotting. [1-3] samples of crude extract from heterotrophically grown *S. obliquus*; [4 and 5] samples of purified RuBPC; [6 and 7] samples of purified PRK.

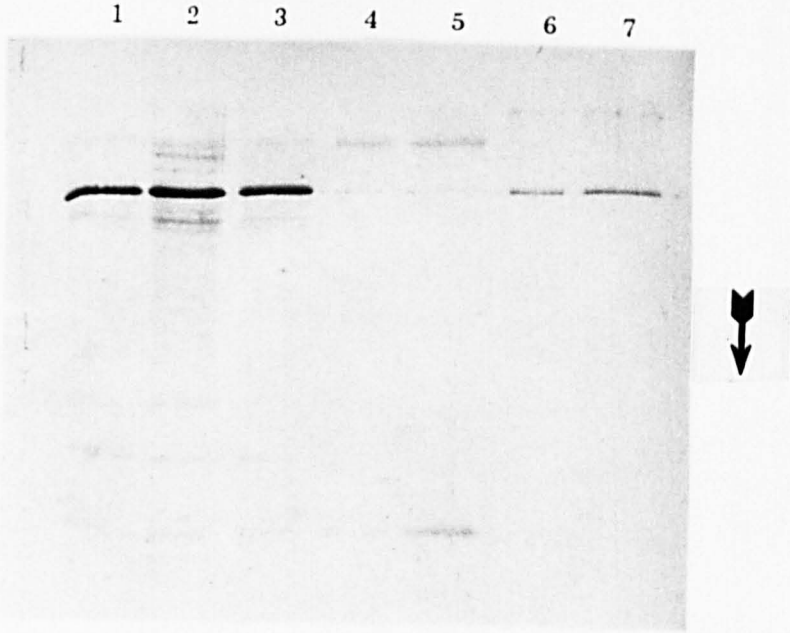
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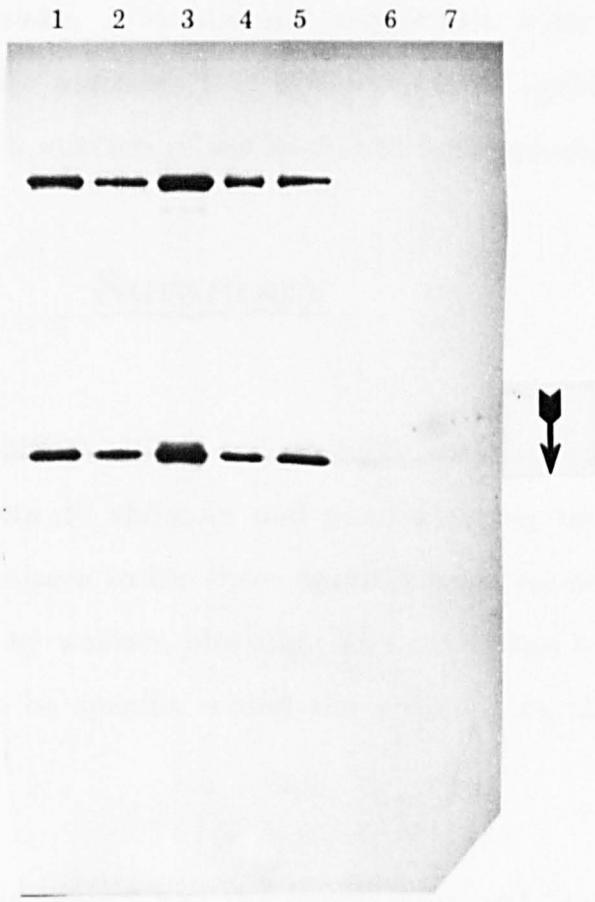
Figure III.18 Western blot using antiserum raised against RuBPC.

The protein samples were subjected to SDS-PAGE prior to blotting. [1-3] samples of crude extract from heterotrophically grown *S. obliquus*; [4 and 5] samples of purified RuBPC; [6 and 7] samples of purified PRK.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could have been considerably reduced, which would have led to the disappearance of the minor peptide bands. The antiserum cross reacted with both subunits.

The antisera raised against either subunit from *S. oleracea* leaf (Nishimura and Akazawa, 1974) did not cross-react with the other subunit. This probably indicates that antisera raised against RuBPC from *S. obliquus* was a mixture of antibodies to both subunits.

Summary

NADPH-dependent G3PDH, PRK and RuBPC were extracted from heterotrophically grown *S. obliquus* and purified using ion-exchange and gel filtration. Antisera to the three enzymes were raised and their specificity was tested by western blotting. The antibodies to PRK and RuBPC were found to be specific whilst the antibody to G3PDH was not.

The next step in the project was the purification and the raising of an antiserum to fructose 1,6-bisphosphate aldolase.

ALDOLASE

Isozymes

A number of enzymes, such as fructose 1,6-bisphosphate (FBP) aldolase, are common to both, the reductive pentose phosphate pathway (RPPP) and glycolysis (Lebherz, *et al.*, 1984). In eukaryotic cells, such enzymes would be expected to be present in both the cytoplasm and the chloroplast. An attempt was made to isolate the aldolase localised in the chloroplast of *Scenedesmus obliquus* since it would be this, rather than the cytoplasmic isozyme, which would have been expected to be a constituent of a multi-enzyme complex participating in the RPPP.

The method used for breaking the cell walls of *S. obliquus* caused considerable damage to the chloroplasts, making it impossible to isolate intact chloroplasts (Graham and Smillie, 1971). As a result, aldolase localised in the chloroplast could not be extracted separately from that found in the cytoplasm.

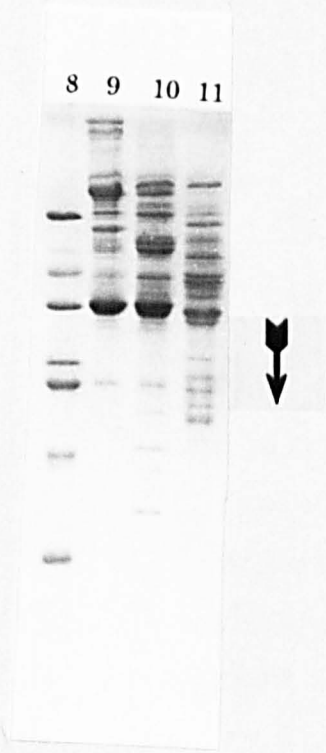
Initial Purification

First attempts to purify aldolase were by the standard chromatography methods in use in the laboratory, namely ion exchange chromatography on Whatman DE-52™, followed by gel filtration on Ultrogel™ and further ion exchange chromatography by FPLC™ on mono Q™.

S. obliquus was grown photoheterotrophically with a view to improving the algal yield obtained from heterotrophically grown alga. Following overnight dialysis, the extract of soluble algal proteins was applied to Whatman DE-52 and proteins were eluted with a linear gradient of phosphate. The fractions with the highest activity of aldolase were pooled, concentrated by ultrafiltration and applied to an Ultrogel AcA-44™ gel filtration column. Fractions eluted from the column subjected to polyacrylamide gel electrophoresis (PAGE) in the presence of SDS showed a need for further purification (Fig. IV.1). The best fractions were again pooled, concentrated and chromatographed on a FPLC mono Q column linked to an FPLC system using a linear gradient of phosphate. The fractions with the highest activity were shown by SDS-PAGE (Fig. IV.2i), to each contain a number of peptide bands, indicating a non-homogeneity of the enzyme preparation. Further chromatography on FPLC mono Q using a much shallower gradient did not remove the contaminating proteins.

Figure IV.1 SDS-PAGE of aldolase eluted from Ultrogel AcA-44.

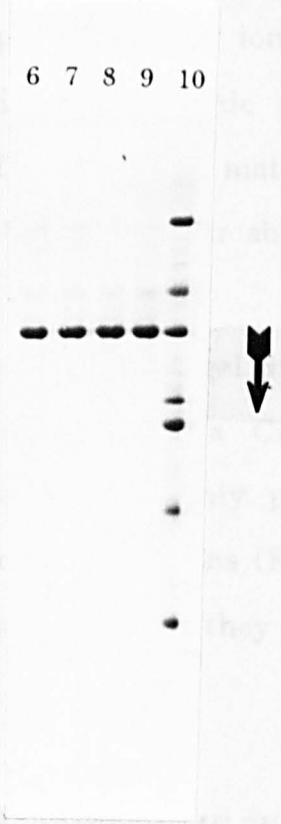
The extract from photoheterotrophically grown *S. obliquus* had previously been purified on Whatman DE-52. [8] M_r marker proteins (66 000, 45 000, 36 000, 29 000, 24 000, 20 000 and 14 200); [9 and 10] peak fractions of aldolase from different gel filtration experiments.



ALDOLASE

Figure IV.2i SDS-PAGE of aldolase eluted from FPLC mono Q.

The extract from photoheterotrophically grown *S. obliquus* had previously been purified on Whatman DE-52 and Ultrogel AcA-44. The protein samples were pooled and concentrated prior to application on the gel. [6] peak fraction of aldolase eluted from FPLC mono Q; [7] best fractions of aldolase from FPLC mono Q were dialysed and reapplied to FPLC mono Q and eluted with a phosphate gradient; [10] M_r marker proteins (66 000, 45 000, 36 000, 29 000, 24 000, 20 000 and 14 200);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Dye-Ligand Chromatography

As these methods had been unsuccessful in purifying aldolase to homogeneity, the possibility of using dye-ligand chromatography was explored. Rabbit liver aldolase had previously been shown to bind to Cibacron blue F3GA in the presence of low ionic strength buffers, from which it was eluted by either high ionic strength, or one of its substrates, FBP (Kido *et al.*, 1980). Gel matrices with a number of covalently-bound dyes were tested for their ability to bind aldolase.

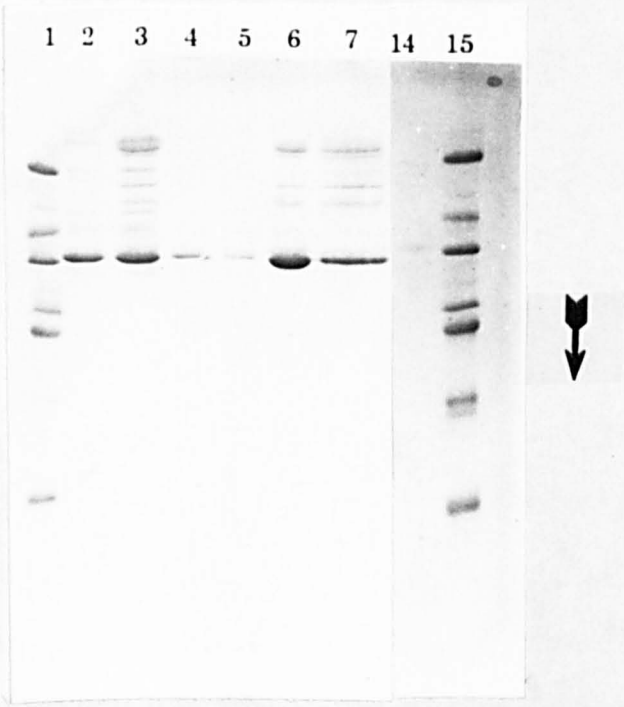
Aldolase previously eluted from Ultrogel did not bind to either an Amicon™ orange or, surprisingly, to a Cibacron™ blue column. Unfortunately, aldolase was not the only protein in the Ultrogel AcA-44 eluant not to bind to these columns (Fig. IV.2ii), and so other dye-ligand columns were tested, to see if they were more successful in binding the enzyme.

Aldolase did bind to Procion™ red HE-7B™, but neither aldolase activity nor protein was eluted even with 2M KCl (Fig. IV.2ii). When FBP was applied to the column, aldolase activity was detected in the eluant. The activities measured were not continuous, and were directly proportional to the concentration of FBP used to wash the column. It was most likely that aldolase bound to the column with the active site free to cleave the FBP used to equilibrate the column. The aldolase assay used (Wu and Racker, 1959) estimated the activity of aldolase (*i.e.* the cleavage of FBP to dihydroxyacetone phosphate

Figure IV.2ii SDS-PAGE of aldolase eluted from dye-ligand chromatography.

The extract from photoheterotrophically grown *S. obliquus* had previously been purified on Whatman DE-52 and Ultrogel AcA-44. [1 and 15] M_r marker proteins (66 000, 45 000, 36 000, 29 000, 24 000, 20 000 and 14 200); [2] unbound protein (Cibacron blue F3GA); [3] unbound protein (Orange dye); [4] FBP-eluted protein (Procion green H4G); [5] unbound protein (Procion green H4G) [6 and 7] KCl-eluted protein (Procion green H4G); [14] FBP-eluted protein (Procion red HE-7B).

ALDOLASE



ALDOLASE

(DHAP) and glyceraldehyde phosphate) by measuring the reduction of DHAP, produced by the enzyme, to glycerol 3-phosphate. Thus, if DHAP was present in the eluant, it would have led to the generation of glycerol phosphate in the assay, until all the DHAP had been utilised, hence mimicking aldolase activity.

Aldolase was also found to bind to a Procion green H-4G column. Activity was eluted by both 0.56mM FBP in a phosphate buffer of ionic strength 0.028 and pH 8.0 and 2M KCl. Surprisingly, aldolase was not the only protein eluted by its substrate (Fig. IV.2ii).

FPLC on Mono-Q

The aldolase eluted from the green dye column with FBP eluant was then subjected to further ion exchange chromatography by FPLC on the mono Q column. The gradient was developed with phosphate buffers of ionic strength 0.028 (pH 8.0) and 0.392 (pH 7.7). Aldolase activity was resolved into three components (Fig. IV.3).

Occurrence of Isozymes

The question now addressed was, did aldolase exist naturally, as three forms in *S. obliquus* or did two arise from a partial post-homogenisation modification of the other, such as *in vitro* protease action?

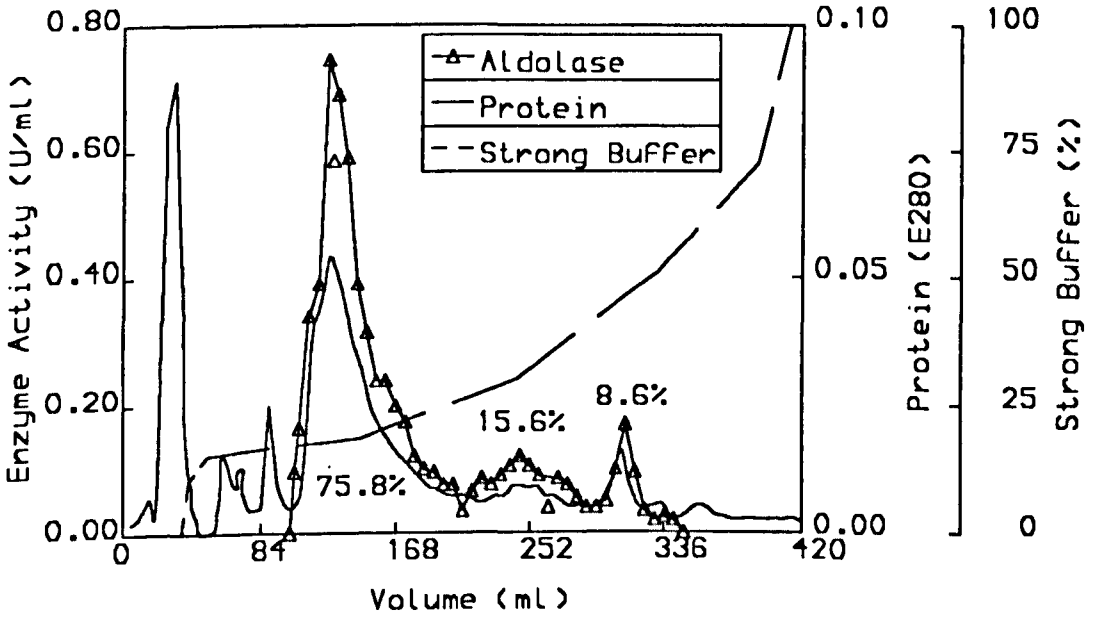


Figure IV.3 Ion-exchange chromatography on FPLC mono Q.

The three forms of aldolase (forms 1-3) were numbered in order of decreasing acidity. The extract from photoheterotrophically grown *S. obliquus* had previously been eluted from Whatman DE-52, Ultrogel AcA-44 and Procion green H4G. The gradient was developed by mixing phosphate buffers of ionic strength 0.028 (pH 8.0) and 0.392 (pH 7.7) on a HR10/10 column with a flow rate of 4mℓ/min. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

When an aldolase fraction previously chromatographed on Whatman DE-52 and Ultrogel AcA-44 was chromatographed on the FPLC mono Q column, the activity was resolved into at least two isozymes (Fig. IV.4). This would suggest that if multiple isozymes were being produced during the purification, it was not occurring during the dye-ligand chromatography step.

Since multiple isozymes of aldolase were only resolved on FPLC mono Q, it was possible that ion exchange on FPLC mono Q could have been responsible for generating artefacts of aldolase. Aldolase that had been previously subjected to chromatography on Whatman DE-52 and Ultrogel AcA-44 and subsequently eluted from the green dye-ligand column with FBP was applied to FPLC mono Q (Fig. IV.5). The first and second isozymes eluted (aldolases -3 and -2, respectively) were pooled separately, concentrated, dialysed and reapplied separately to FPLC mono Q (Figs. IV.6 and IV.7). Each isozyme was eluted at an identical concentration to that required for the corresponding original elution. There was no evidence from the elution profile, of an interconversion during the intervening period between the two ion exchange steps.

The possibility of multiple isozymes being formed as a result of post-homogenisation proteolytic action would appear unlikely, as azo-casein hydrolysis (a measure of protease activity) was shown to occur only at a very low rate in the crude algal extract (Fig. IV.8).

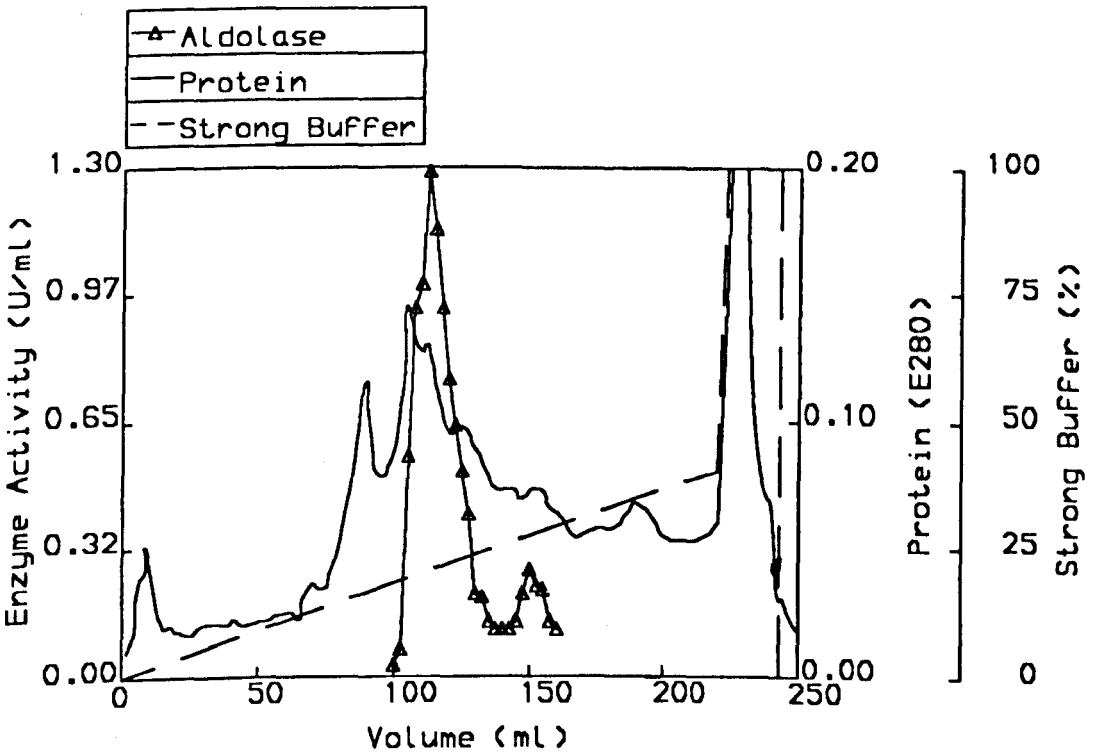


Figure IV.4 Ion-exchange chromatography on FPLC mono Q.

Only the two most basic forms, aldolase-2 and aldolase-3 were assayed. The extract from photoheterotrophically grown *S. obliquus* had previously been purified on Whatman DE-52, and Ultrogel AcA-44. The gradient was developed by mixing phosphate buffers of ionic strength 0.028 (pH 8.0) and 0.392 (pH 7.7) on a HR10/10 column with a flow rate of 4mℓ/min. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

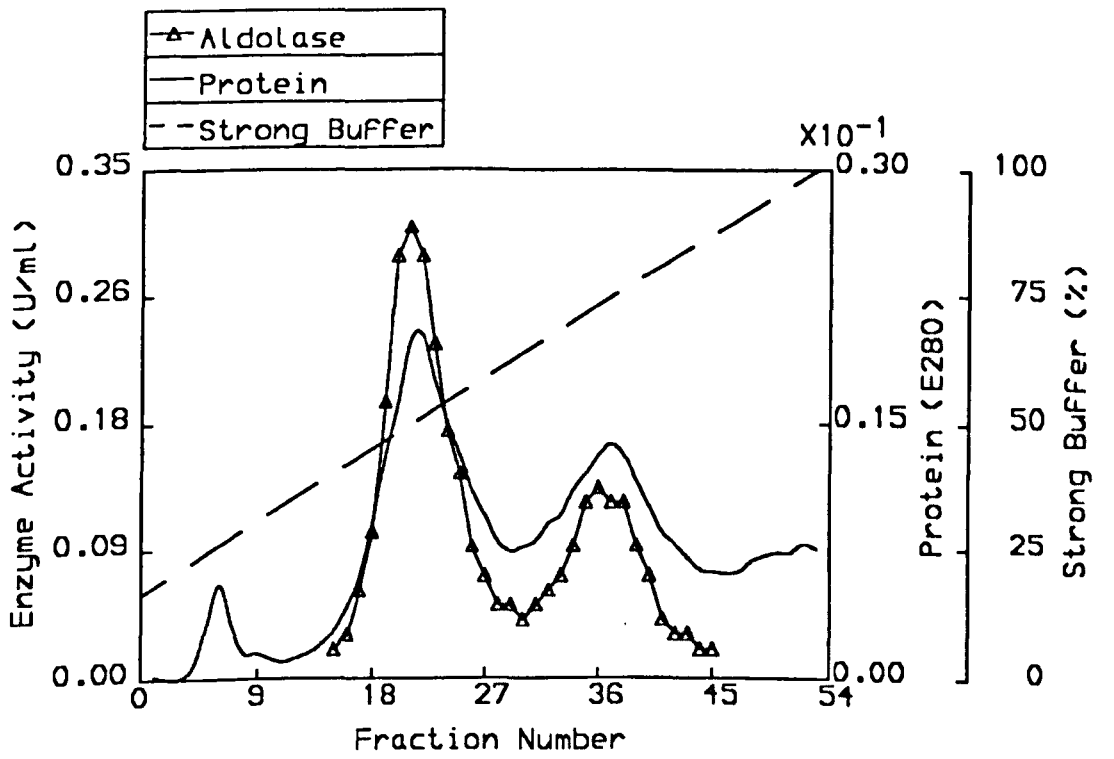


Figure IV.5 Ion-exchange chromatography on FPLC mono Q of aldolase.

Only the two most basic forms, aldolase-2 and aldolase-3 were assayed. The extract from photoheterotrophically grown *S. obliquus* had previously been eluted from Whatman DE-52, Ultrogel AcA-44 and Procion green H4G. The gradient was developed by mixing phosphate buffers of ionic strength 0.028 (pH 8.0) and 0.392 (pH 7.7) on a HR10/10 column with a flow rate of 4mℓ/min. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

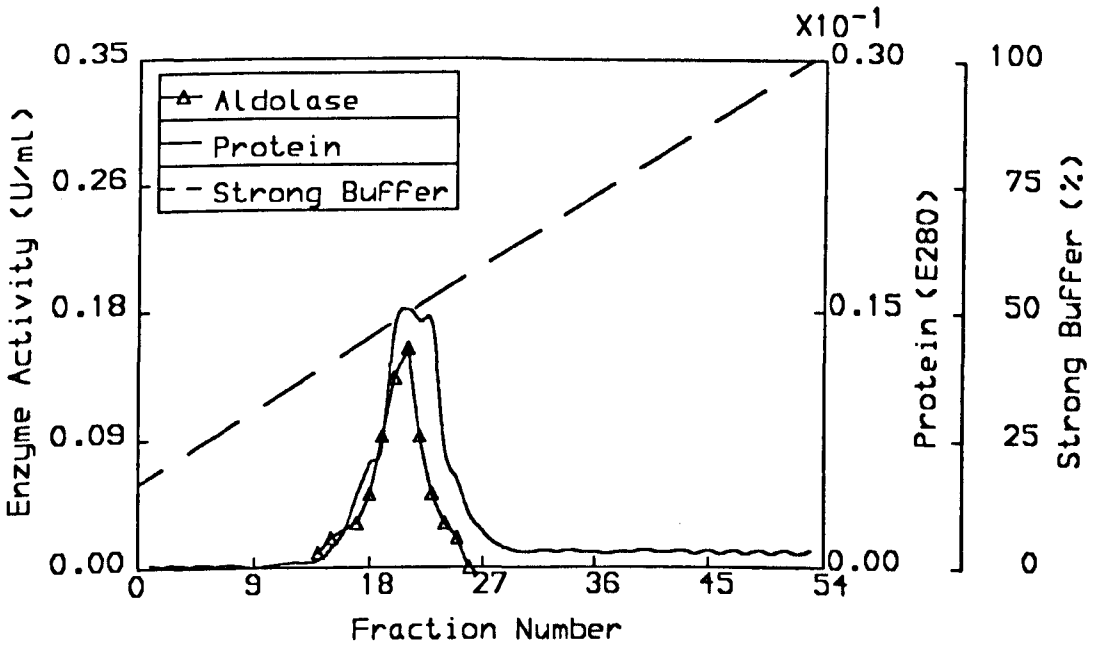


Figure IV.6 Ion-exchange chromatography on FPLC mono Q of aldolase.

Basic isozyme (aldolase-3) from previous chromatography on FPLC mono Q (Fig. IV.5) was reapplied to FPLC mono Q. Only the two most basic forms, aldolase-2 and aldolase-3 were assayed. The gradient was developed by mixing phosphate buffers of ionic strength 0.028 (pH 8.0) and 0.392 (pH 7.7) on a HR10/10 column with a flow rate of 4mℓ/min. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

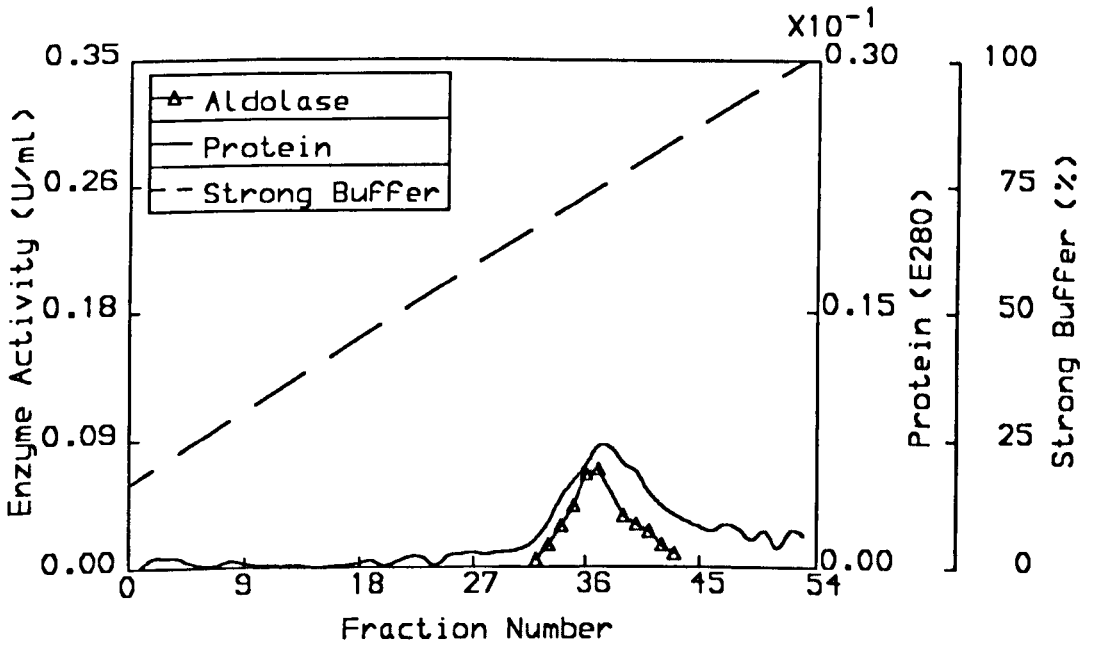


Figure IV.7 Ion-exchange chromatography on FPLC mono Q of aldolase.

Acidic isozyme (aldolase-2) from penultimate chromatography on FPLC mono Q (Fig. IV.5) was reapplied to FPLC mono Q. Only the two most basic forms, aldolase-2 and aldolase-3 were assayed. The gradient was developed by mixing phosphate buffers of ionic strength 0.028 (pH 8.0) and 0.392 (pH 7.7) on a HR10/10 column with a flow rate of 4mℓ/min. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

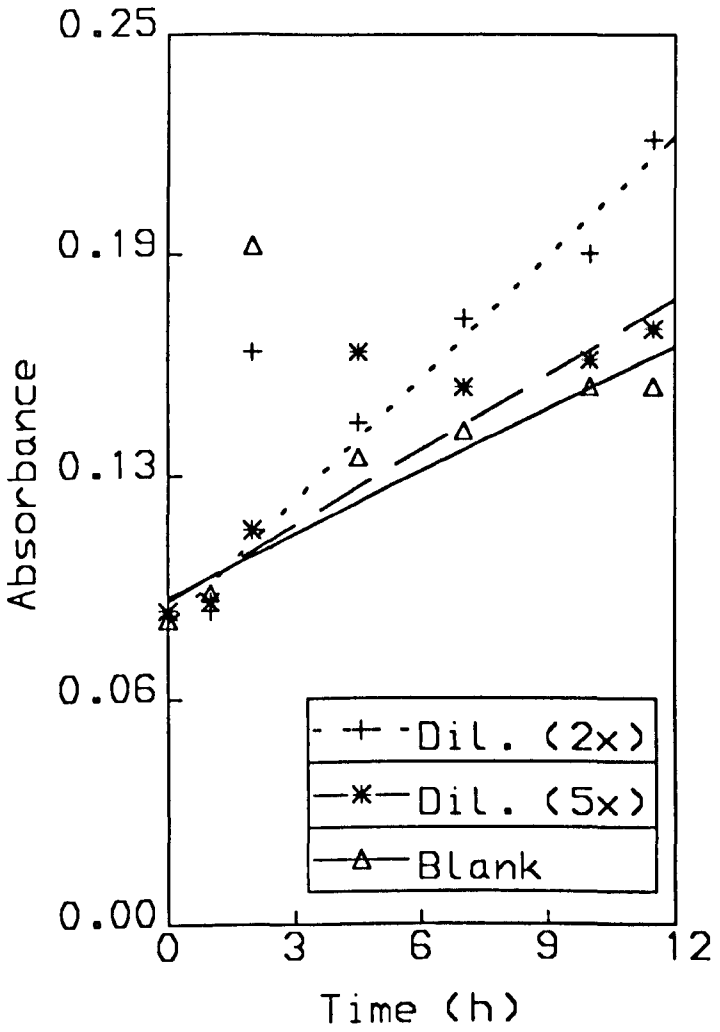


Figure IV.8 Estimation of protease activity in crude extract from *S. obliquus*.

The extract was obtained from heterotrophically grown *S. obliquus* and diluted two- and five-fold, prior to assaying. The blank did not contain any crude extract. The absorbance was measured at 340nm.

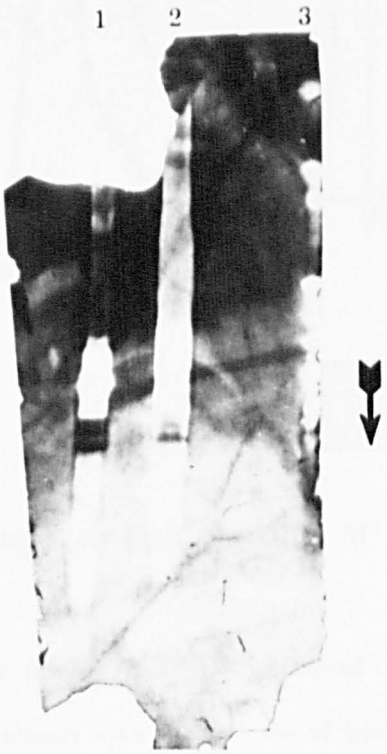
Samples of aldolase were isoelectrically focused, the presence of aldolase being detected by the use of a positive activity stain. Since DTT caused a darkening of the whole gel, reduced glutathione, a milder reducing agent, was substituted. A zymogram with dark bands on a lighter background was expected (Spencer *et al.*, 1964), but surprisingly, the background was still stained and the protein bands, excluding aldolase, were visible as much lighter bands (Fig. IV.9). At least two bands were visible in the autotrophic crude extract, whilst only one was visible in the heterotrophic extract. Thus, the variation in the isozyme profiles was shown to occur, prior to employing any purification procedures.

Phosphate buffers were found to be essential for the resolution of aldolase activity during chromatography on FPLC mono Q. When FBP-eluted aldolase from green dye-ligand chromatography was subjected to ion exchange on FPLC mono Q using Tris-HCl buffers, only a single peak of aldolase activity, associated with the major protein peak was observed (Fig. IV.10). However, following dialysis of this aldolase preparation into phosphate and re-application to FPLC mono Q, the expected aldolase isozymes were resolved when elution was performed with a phosphate gradient (Fig. IV.11). The conductivity of the Tris-HCl equilibrating and strong buffers were comparable to the corresponding phosphate buffers (Table IV.1), thus, phosphate appears to differentially elute the various isozymes of aldolase from FPLC mono Q.

Figure IV.9 Isoelectric focusing of crude extract.

Gel developed with aldolase activity stain. [1] crude extract from autotrophically grown *S. obliquus*; [2] crude extract from heterotrophically grown *S. obliquus*; [3] pI marker proteins (10.60, 8.30, 7.30, 6.45, 5.90, 5.65 and 4.85).

ALDOLASE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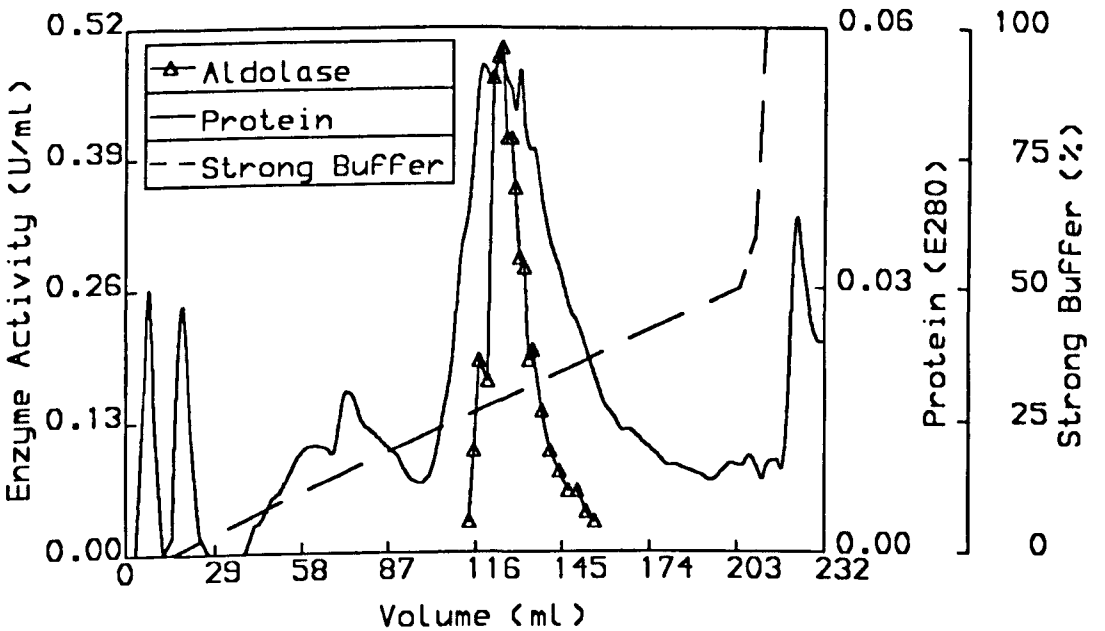


Figure IV.10 Ion-exchange chromatography of aldolase on FPLC mono Q.

The gradient was developed by mixing Tris-HCl buffers of ionic strength 0.025 and 0.392 at pH 8.5 on a HR10/10 column with a flow rate of 4mℓ/min. The extract from autotrophically grown *S. obliquus* had previously been purified on Whatman DE-52, Ultrogel AcA-44 and Procion green H4G. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

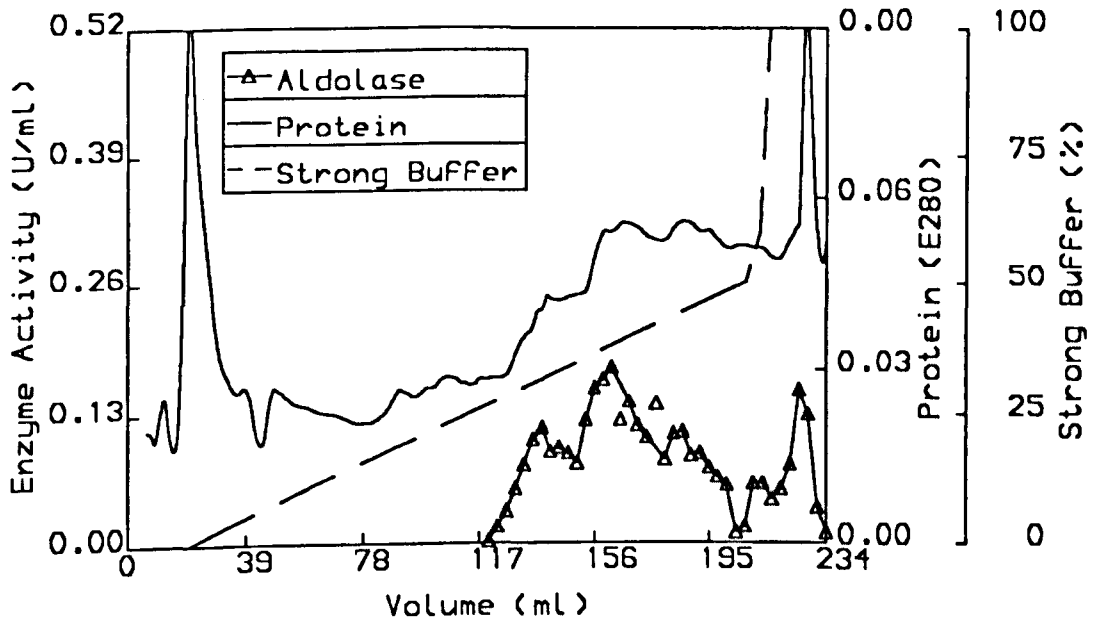


Figure IV.11 Ion-exchange chromatography of aldolase on FPLC mono Q.

All fractions containing aldolase activity from previous chromatography on FPLC mono Q (Fig. IV.10) were pooled, dialysed against a phosphate buffer of ionic strength 0.028 of pH 8.0 concentrated and reapplied to FPLC mono Q. The gradient was developed by mixing phosphate buffers of ionic strength 0.028 (pH 8.0) and 0.392 (pH 7.7) on a HR10/10 column with a flow rate of 4mℓ/min. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

Buffer	Conductivity (mS)	
	Phosphate	Tris-HCl
Equilibrating	2.85	2.15
Strong	19.50	19.50

Table IV.1 Conductivity of phosphate and Tris-HCl buffers used for the purification of aldolase and phosphoglycerate kinase (PGK).

Cellular Localisation of Isozymes

Having determined that multiple isozymes of aldolase were present *in vivo* in *S. obliquus*, it was extremely likely that at least one of the isozymes was localised in the chloroplast. Since the alga had been grown photoheterotrophically, the predominant isozyme would be expected to be the cytoplasmic aldolase.

By means of SDS-PAGE, all the aldolase isozymes isolated from *S. obliquus* were shown to have subunits of 40 000 (Fig. IV.12). This is in contrast to the aldolases from the leaves of *Spinacia oleracea* (Lebherz *et al.*, 1984; Jacobshagen and Schnarrenberger, 1988) or from *Pisum sativum* (Anderson *et al.*, 1975) or even the green alga *Chara foetida* (Jacobshagen and Schnarrenberger, 1988), where the chloroplast aldolase was characterised by a smaller subunit.

The molecular weight of all three native aldolase isozymes was estimated by gel filtration to be 109 000 (Fig. IV.13). This value is much lower than the molecular weights published for aldolase from the leaf of *P. sativum* (Anderson *et al.*, 1975), *Zea mays* (Gasperini and Pupillo, 1982/83) or wheat (Murphy and Walker, 1981). The implication of the molecular weight studies of the aldolase from *S. obliquus* was that it was present in the extract as a trimer. The aldolases from *S. obliquus* were not sensitive to metal ions and were thus classed as type I (Lebherz and Rutter, 1975). Since all other type I aldolases characterised were tetramers (Anderson *et al.*, 1975), it was highly unlikely that *S. obliquus* would possess the only trimeric type I aldolase. This discrepancy was attributed to the inherently limited accuracy of using gel filtration in the determination of the molecular weight of undenatured proteins (Bio-Rad, 1971). In fact, Winzor and Scheraga (1963) discovered that the migration of proteins frequently depended on the protein concentration. Nevertheless, it was a rapid and simple method for obtaining approximate values, whilst purifying proteins.

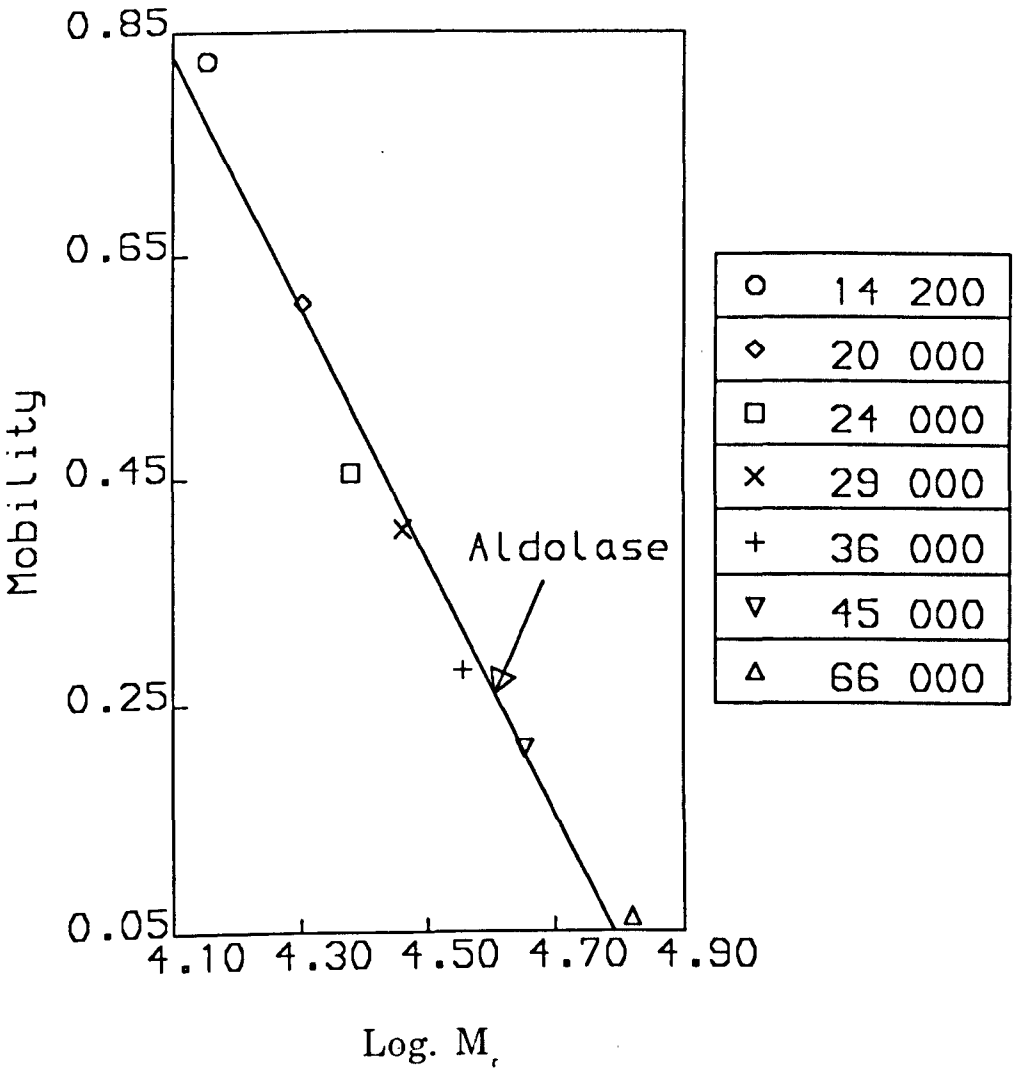


Figure IV.12 M_r determination of aldolase subunit by SDS-PAGE.

The relative mobilities were measured from the gel and the above plot was used to obtain their M_rs.

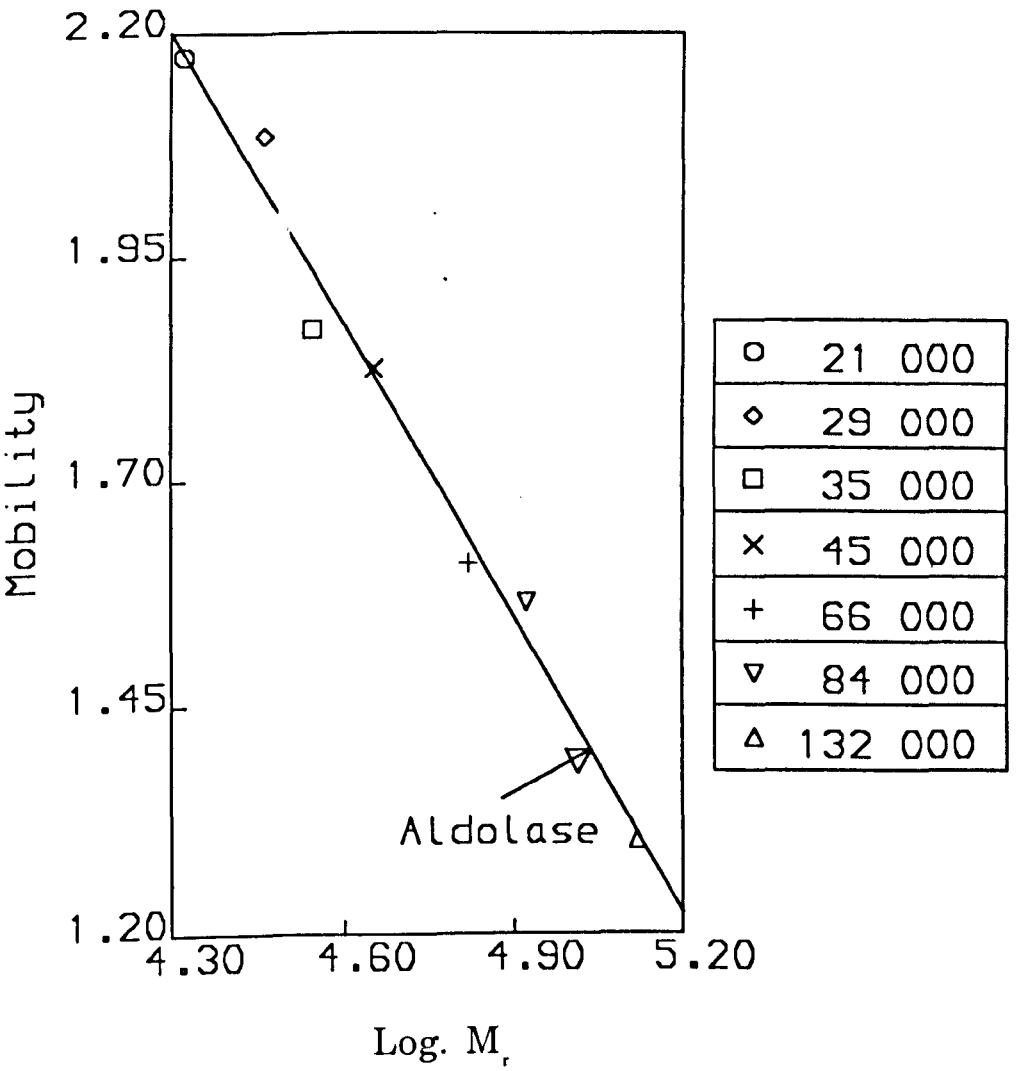


Figure IV.13 M_r determination of native aldolase from Ultrogel AcA-44™.

The mobilities were measured from the elution profile and the above plot was used to obtain their M_rs.

For the algal aldolase isozymes, aldolase-3 had a much higher specific activity (14.9) than aldolase-2 (5.5U (mg protein)$^{-1}$). This was consistent with a cytoplasmic origin for aldolase-3, since both Lebherz *et al.* (1984) and Jacobshagen and Schnarrenberger (1988) showed that in purified aldolase preparations from *S. oleracea* leaf and *C. foetida*, respectively, the specific activity of the chloroplast aldolase was considerably lower than that of the cytoplasmic isozyme.

Lebherz *et al.* (1984) discovered that the cytoplasmic aldolase from *S. oleracea* leaf was stable to prolonged heating at 50°C, whilst the chloroplast isozyme was inactivated by this treatment. In the case of the algal isozymes, aldolase-2 was found to be more susceptible to thermal denaturation than aldolase-3 (Fig. IV.14). This finding lent further credence to the notion that aldolase-2 was the chloroplast aldolase, whilst aldolase-3 was localised in the cytoplasm.

Further evidence for the cellular distribution may be obtained from the study of aldolase found in autotrophic *S. obliquus*, since it had previously been discovered that the chloroplast aldolase was the dominant isozyme in *Chlamydomonas mundana* (Russell and Gibbs, 1967), only when the organism was grown autotrophically.

Autotophically grown *S. obliquus* cells were extracted in the usual way, chromatographed as previously described and the eluant from the Procion green column applied to FPLC mono Q. The elution profile on FPLC mono Q showed that aldolase 3, which was the predominant isozyme, when heterotrophic cells were used (Fig. IV.3), now accounted

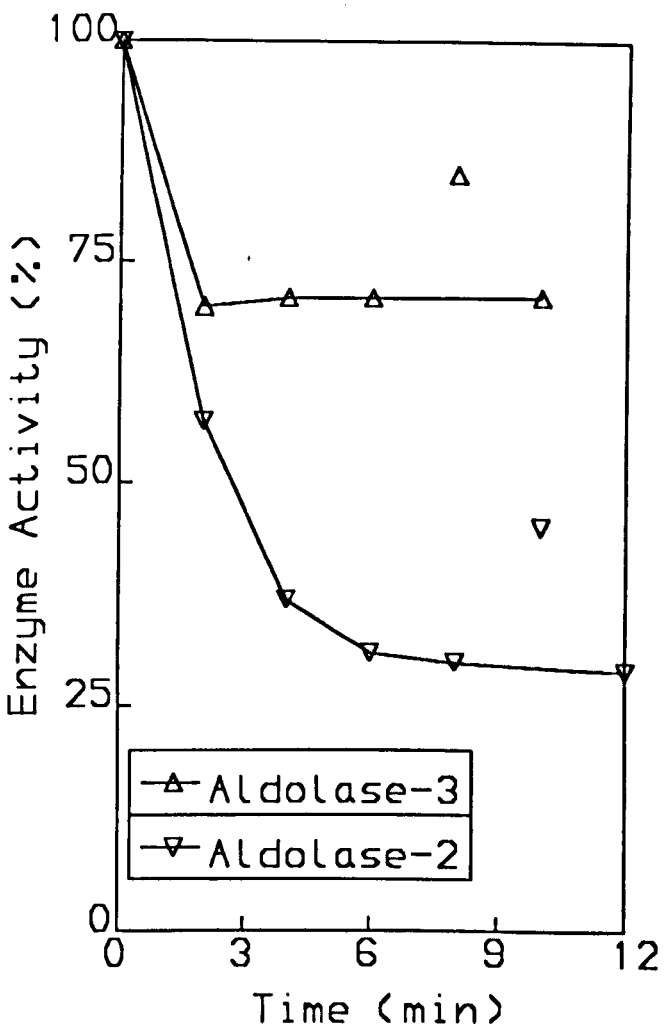


Figure IV.14 The thermal stability of aldolase isozymes.

Since the protein content of the aldolase-3 preparation was approximately twice that of aldolase-2, aliquots of aldolase-3 were diluted two-fold prior to heating. Aliquots of enzyme were heated to 50°C in a water bath between 0-12min. This experiment was based on that carried out by Lebherz *et al.* (1984).

for less than a quarter of the total aldolase activity (Fig. IV.15). This was consistent with the suggestion that aldolase-3 was the cytoplasmic enzyme functioning in glycolysis and gluconeogenesis.

The other two isozymes whilst representing a quarter of the total aldolase isolated from heterotrophic cells (Fig. IV.3), made up approximately three quarters of the total aldolase activity isolated from autotrophically grown cells (Fig. IV.15). Since aldolases -2 and -1 were induced by photosynthetic activity, they were considered to be involved in the RPPP and hence localised in the chloroplast. Two isozymes of chloroplast aldolase have previously been detected in extracts from leaves of *Z. mays* (Valenti *et al.*, 1987) and *P. sativum* (Anderson and Pacold, 1972). The changes in the protein profile in the extracts from *S. obliquus* indicated that the requirements of photosynthesis could be met by:

1. the *de novo* synthesis of the second and third isozymes;
2. a structural modification of the first to yield the other two;
3. the inhibition of the synthesis of the first isozyme;
4. or a combination of any of the above.

Murphy and Walker (1981) believed that the sedoheptulose 1,7-bisphosphate (SBP)-aldolase activity (*i.e.* the condensation of erythrose 4-phosphate and DHAP to SBP) was catalyzed by the same molecule as that catalysing the FBP aldolase activity in the chloroplast of wheat leaves. Nevertheless, it is quite possible, that in *S. obliquus*, the two reactions may not preferentially, be catalysed by

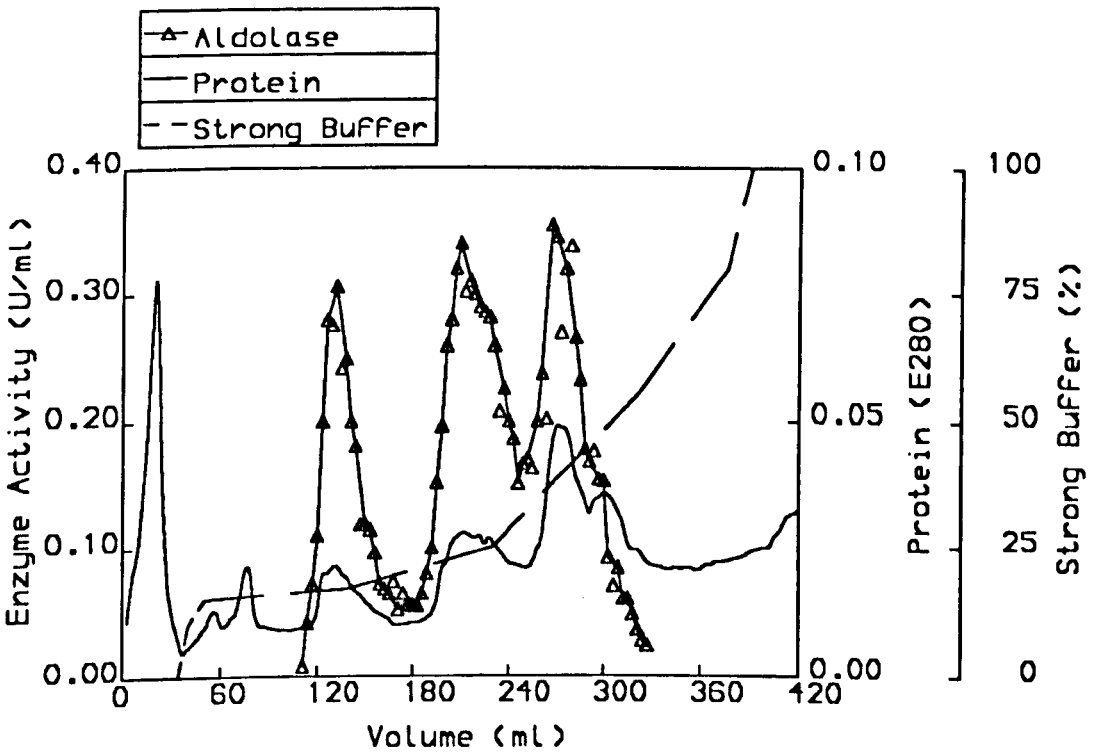


Figure IV.15 Ion-exchange chromatography of aldolase on FPLC mono Q.

The extract from autotrophically grown *S. obliquus* had previously been purified on Whatman DE-52, Ultrogel AcA-34 and Procion green H4G. The gradient was developed by mixing phosphate buffers of ionic strength 0.028 (pH 8.0) and 0.392 (pH 7.7) on a HR10/10 column with a flow rate of 4mℓ/min. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

the same molecule. Thus aldolases -2 and -1 could have been the chloroplast FBP and SBP aldolases. Since the commercial preparation of SBP, a substrate for SBP-aldolase was contaminated with FBP (Sigma, 1990), it was not possible to confirm the nature of these two aldolases, which are dominant in autotrophically grown alga.

Having taken into account the differences in specific activities and thermal stabilities of the first two isozymes, together with the elution profiles on the FPLC mono Q of both photoheterotrophic and autotrophic grown *S. obliquus*, it is strongly believed that the first isozyme was localised in the cytoplasm, whilst the remaining two isozymes were from the chloroplast.

Quantification of Aldolase Activity

Unfortunately, due to the extremely tough cell walls of *S. obliquus*, the degree of breakage of batches of the alga was found to be inconsistent. This variation was found to be even more pronounced when breakage of algal cells grown under different conditions was examined. In order to quantify the release of aldolase from algal cells, an attempt was made to find an internal marker which would enable the degree of cell breakage to be estimated. Mitochondrial cytochrome c was originally used. This protein was estimated in algal crude extracts from its difference spectrum (Fig. IV.16). However, there did not appear to be any correlation between cytochrome c and either PGK

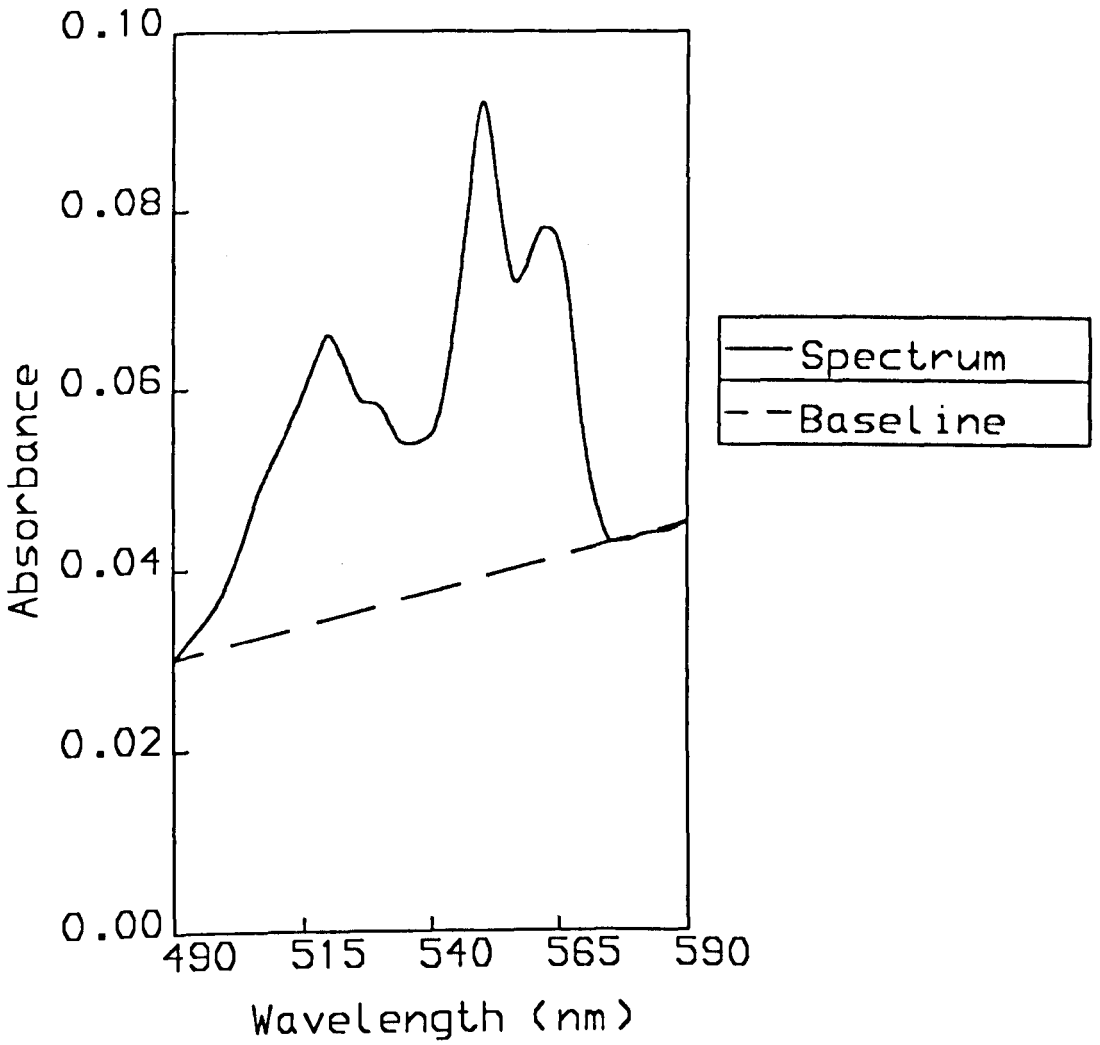


Figure IV.16 Oxidised versus reduced difference spectrum of cytochrome c in crude extract from *S. obliquus*.

The crude extract was concentrated by ultrafiltration, prior to estimation. Oxidation and reduction were carried out by the addition of potassium ferricyanide and sodium dithionite, respectively

or aldolase in a number of extracts isolated from heterotrophically grown cells.

Whilst searching for a marker for cell breakage, it was discovered that the ratio of aldolase to PGK in extracts depended on the manner under which *S. obliquus* was grown. The ratio was high (21.0) when the alga was grown photoheterotrophically and low (1.0) under autotrophic conditions. As no measurement was made of the degree of cellular breakage during enzyme extraction, it was not possible to determine whether the decrease in the aldolase to PGK activity ratio under autotrophic growth was due to:

1. an increase in aldolase activity;
2. a decrease in PGK activity;
3. or a combination of both.

Nevertheless, it seems reasonable to assume that proteins are synthesised during adaptation to autotrophic growth (Russell and Gibbs, 1967).

Purification of Aldolase-2

The dominant aldolase present in autotrophic *S. obliquus* was purified and an antibody raised against it.

Two batches of autotrophically grown *S. obliquus* suspended in a phosphate buffer of ionic strength 0.130 and pH 7.5, broken and the extracts combined. After overnight dialysis, the combined extract was subjected to ion exchange chromatography on Whatman DE-52 (Fig. IV.17). All fractions containing aldolase activity were pooled, concentrated and applied to Ultrogel AcA-34™ in two portions (Fig. IV.18).

The eluted aldolase activity was pooled, concentrated, divided into ten portions and each portion chromatographed on Procion green H-4G. The bound aldolase was eluted with 0.56mM FBP in a phosphate buffer of ionic strength 0.028 and pH 8.0. (Table IV.2). The eluants from all ten chromatographic runs were pooled, concentrated and applied to the FPLC mono Q column.

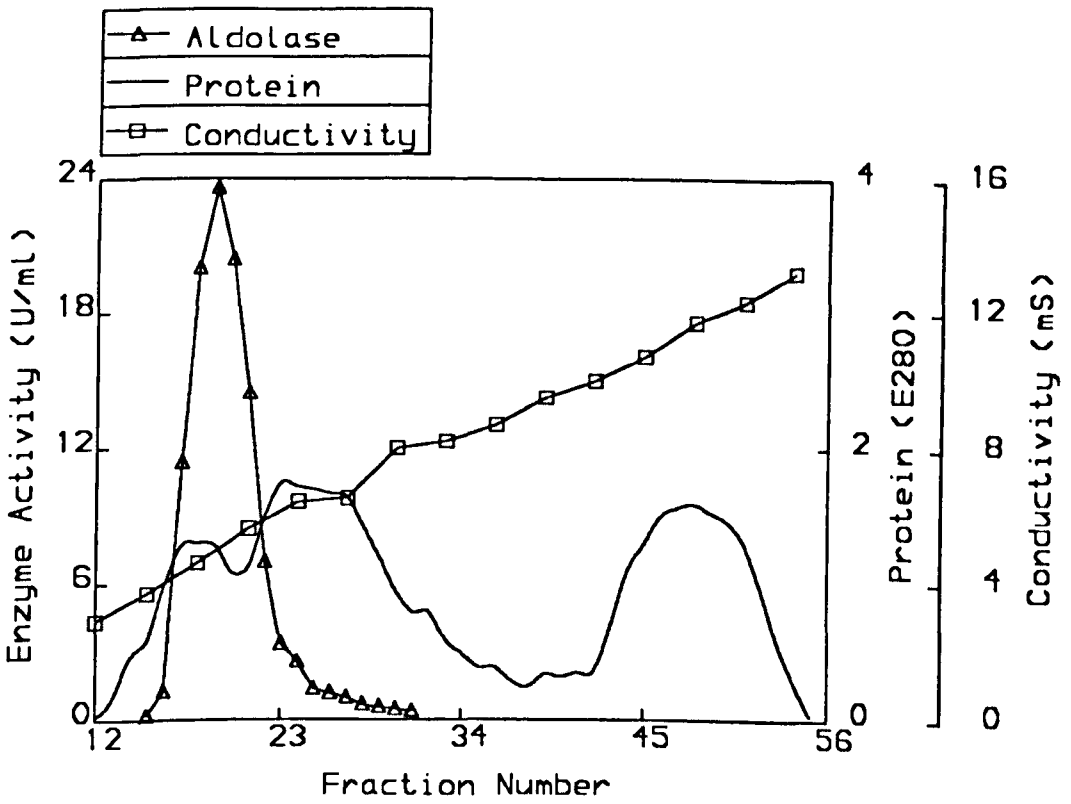


Figure IV.17 Ion-exchange chromatography of crude extract from autotrophically grown *S. obliquus* on Whatman DE-52.

The gradient was developed by mixing phosphate buffers of ionic strength 0.028 (pH 8.0) and 0.392 (pH 7.7) on a 2.2 × 29.5cm column with a flow rate of 50mℓ/h. Fractions of 6mℓ were collected. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

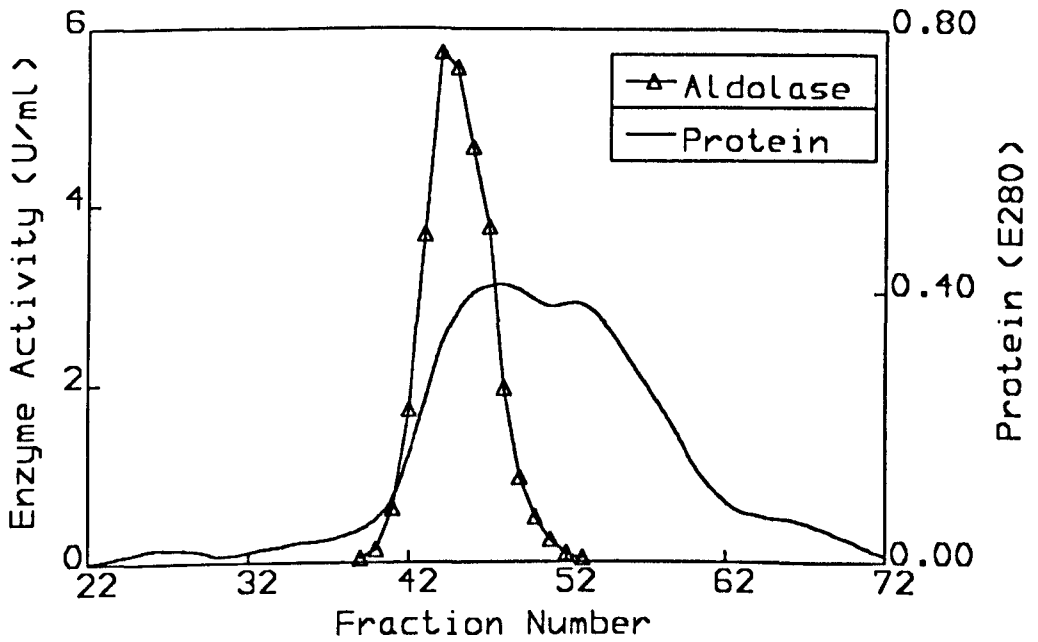


Figure IV.18 Gel filtration of aldolase on Ultrogel AcA-34.

Elution was performed on a 2.6 × 83.0cm column at pH 8.0 with a flow rate of 20mℓ/h. The extract from autotrophically grown *S. obliquus* had previously been purified on Whatman DE-52. The eluting buffer was phosphate with an ionic strength of 0.028 and supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol. Fractions of 3mℓ were collected.

Run	Activity Applied (U)	Activity Eluted (U)	Yield (%)
i	9.90	7.93	80.1
ii	11.14	11.61	104.2
iii	11.14	8.50	76.3
iv	11.14	8.11	72.8
v	11.14	8.11	72.8
vi	11.14	8.22	73.8
vii	12.38	8.40	67.9
viii	8.66	6.11	70.6
ix	9.90	5.73	57.9

Table IV.2 Dye-ligand chromatography of aldolase on Procion green H4G.

The extract from autotrophically grown *S. obliquus* was purified on Whatman DE-52 and Ultrogel AcA-34, prior to application on H4G. Elution was performed on a 15mℓ column at pH 7.5 with a flow rate of 20mℓ/h. The eluting buffer was phosphate with an ionic strength of 0.050 and supplemented with 12mM PGA, 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol. The PGA-eluted protein was collected in a single fraction of 20mℓ

The initial chromatography using a complicated gradient, to save time, did resolve the algal aldolase into three components (Fig. IV.15). However, a much better separation was obtained by re-chromatographing the total aldolase, after concentration and dialysis, using a much simpler gradient (Fig. IV.19). It was now more obvious that in autotrophically grown *S. obliquus*, aldolase-2 was the predominant isozyme. All three isozymes, upon elution from the first FPLC mono Q step, were shown by SDS-PAGE (Fig. IV.20) to be completely pure.

The details of the purification are shown in Table IV.3. Although a low yield of 3.6% was obtained, it was considerably higher than that of 0.14% achieved by Hatz and Leuthardt (1967) from *Pisum sativum*.

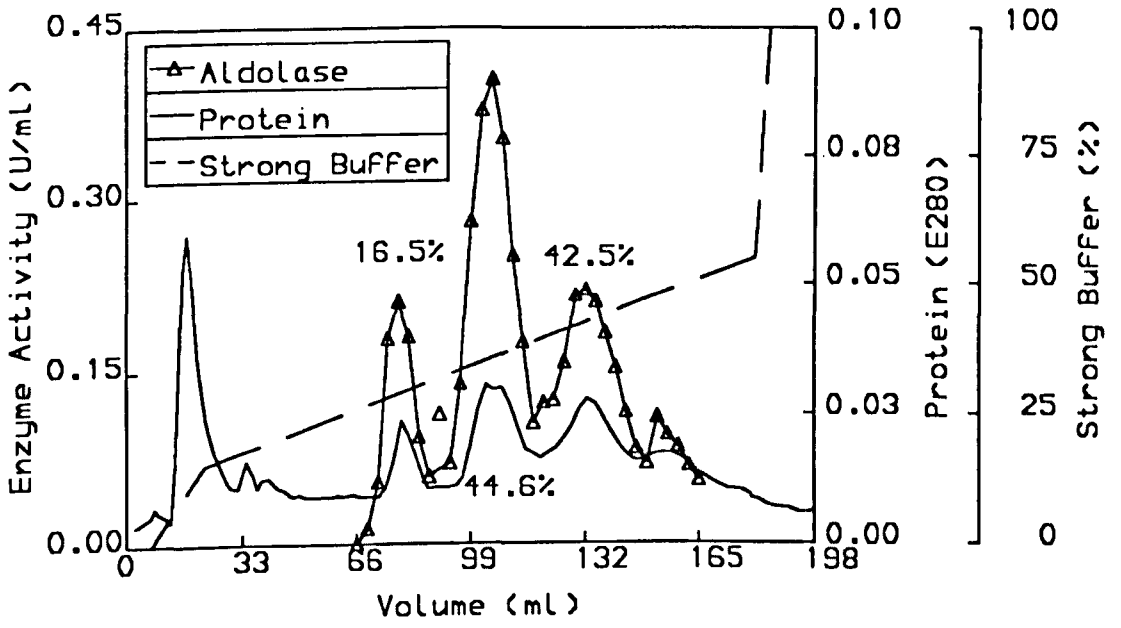
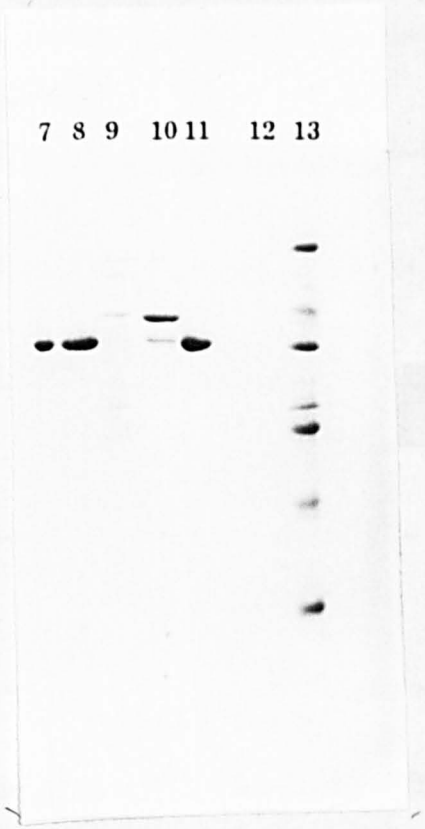


Figure IV.19 Ion-exchange chromatography of aldolase on FPLC mono Q.

All fractions containing aldolase activity from chromatography on FPLC mono Q (Fig. IV.15) were pooled, dialysed against a phosphate buffer of ionic strength 0.028 and pH 8.0, concentrated and reapplied to FPLC mono Q. A simple gradient was developed by mixing phosphate buffers of ionic strength 0.028 (pH 8.0) and 0.392 (pH 7.7) on a HR10/10 column with a flow rate of 4mℓ/min. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

Figure IV.20 SDS-PAGE of purified aldolase.

Extract from autotrophically grown *S. obliquus* was purified on Whatman DE-52, Ultrogel AcA-34, Procion green H4G and FPLC mono Q, and then concentrated prior to application on gel. [7] aldolase-3; [8] aldolase-2; [11] aldolase-1; [13] M_r marker proteins (66 000, 45 000, 29 000, 29 000, 24 000, 20 000 and 14 200).



ALDOLASE

Step	Total Activity (U)	Protein Conc. (mg/mℓ)	Enzyme Activity (U/mℓ)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Crude Extract	922.2	8.26	0.96	0.12	1.0	100.0
DE-52	415.0	3.84	2.26	0.59	5.2	45.3
AcA-34	304.2	3.11	5.53	1.70	14.7	33.1
H4G	63.1	0.08	0.35	4.32	37.4	6.9
mono Q (i)	33.8	0.03	0.15	4.90	42.2	3.6
mono Q (ii)	2.1	0.02	0.10	5.50	47.4	0.2

Table IV.3 Purification profile of aldolase-3 from autotrophically grown *S. obliquus*.

Western Blotting

Aldolase-2 from the second FPLC mono Q chromatography was used to raise an antibody against chloroplast aldolase.

Western blotting of SDS-PAGE (Fig. IV.21) indicated that the antibody raised against aldolase-2, cross-reacted with algal aldolases -3 and -1, but did not cross-react with any other proteins in the soluble crude extract from the alga, although multiple bands were detected by the blot at the top of each track containing protein. It appeared as if a high molecular weight peptide, with the ability to bind the antibody was present in all the protein preparations used in the blot, including a purified commercial preparation of rabbit muscle aldolase. It was more likely an artefact of that particular blotting.

Iso-electric focusing was performed on the three aldolases. The aldolase bands being detected by western blotting (Fig. IV.22), since this had a much greater sensitivity than Coomassie blue for aldolase detection. Hence, much less protein could be applied in iso-electric focusing, leading to narrower bands and hence, better resolution. The pI's of the three aldolases were determined with a set of coloured marker proteins (Fig. IV.23). The pI's of the three isozymes are given in Table IV.4.

Figure IV.21 Western blot using antiserum raised against aldolase-2. Protein samples were subjected to SDS-PAGE prior to blotting. [1 and 2] two samples of extract from autotrophically grown *S. obliquus*; [3] commercial preparation of rabbit muscle aldolase; [4] partially purified PGK; [5] extract from autotrophically grown *S. obliquus* eluted from Whatman DE-52 and Ultrogel AcA-44; [6] purified aldolase-3; [7] purified aldolase-2; [8] purified aldolase-1; concentrated prior to application on gel.

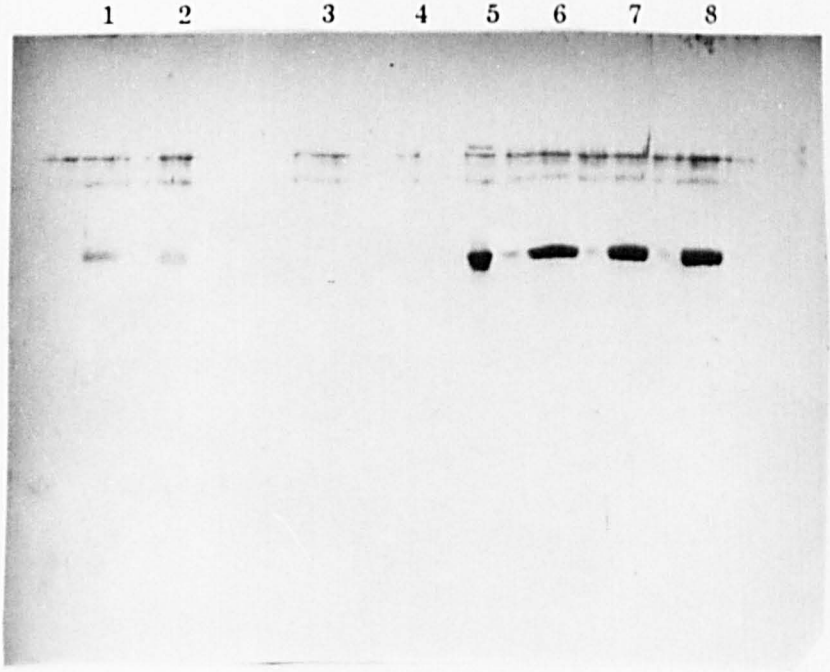

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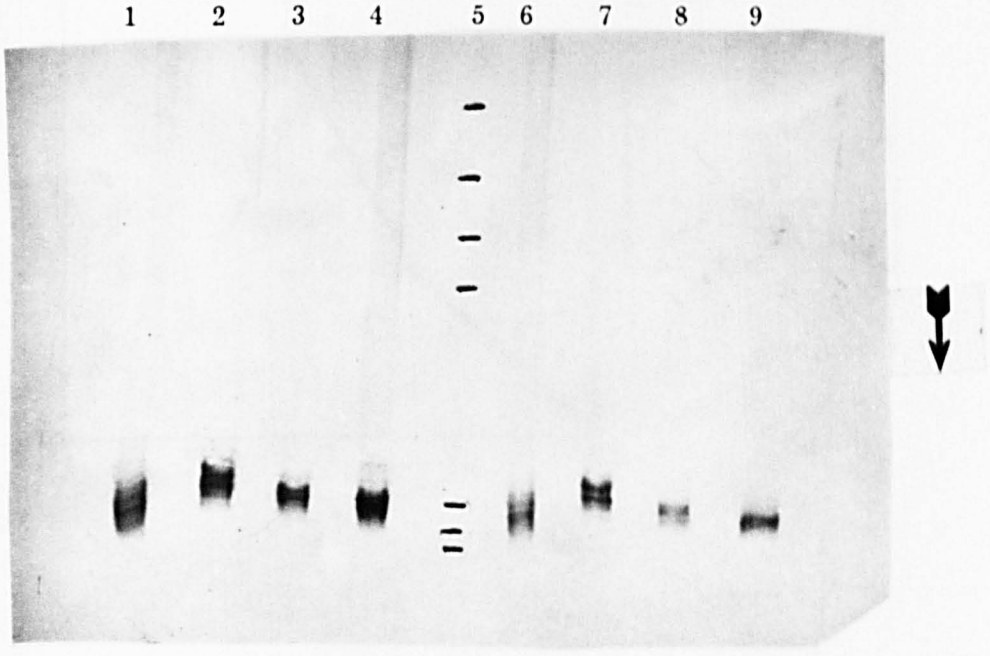
Figure IV.22 Western blot using antiserum raised against aldolase-2. Protein samples were isoelectrically focused prior to blotting. Positions of pI protein markers etched with pencil prior to developing blot. [1 and 6] crude extract from autotrophically grown *S. obliquus*; [2 and 7] aldolase-3; [3 and 8] aldolase-2; [4 and 9] aldolase-1; [5] pI protein markers (10.60, 8.30, 7.30, 6.45, 5.90, 5.65 and 4.85). 2μℓ of samples were loaded in tracks 1-4 and 1μℓ in tracks 6-9.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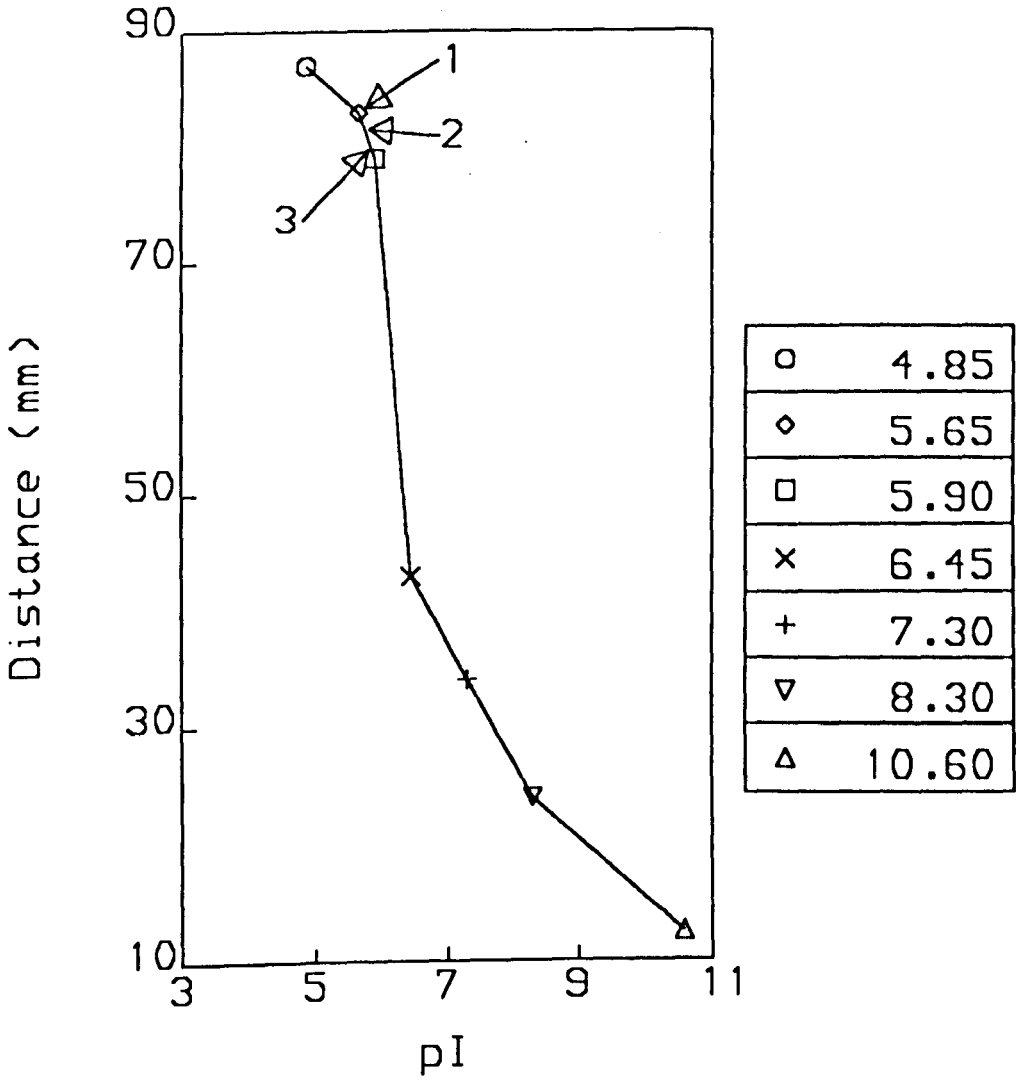


Figure IV.23 Determination of the isoelectric points of aldolase isozymes.

The relative mobilities were measured from the gel and the above plot was used to obtain their isoelectric points. The arrows indicate the three positions of the three forms of aldolase.

Isozyme	Isoelectric Point (pI)	
	S. obliquus	*Z. mays* Leaf
1	5.75	4.20
2	5.80	4.60
3	6.05	6.70

Table IV.4 Comparison of the isoelectric points of aldolase isozymes from *S. obliquus* and *Z. mays* Leaf.

The .S. obliquus was grown autotrophically whilst the values for *Z. mays* were obtained from Valenti *et al.* (1987).

Summary

Aldolase was extracted from autotrophically grown *S. obliquus* and purified using ion exchange, gel filtration and dye ligand chromatography. It was resolved into three components during FPLC on mono Q, and indirect methods hinted that the two acidic forms were localised in the chloroplast. An antibody was raised against one of the acidic forms, which although was specific for aldolase, was found to cross react with the other two forms.

PHOSPHOGLYCERATE KINASE

Search for Isozymes

Phosphoglycerate kinase (PGK) would be expected, like fructose 1,6-bisphosphate (FBP) aldolase, to be localised in both, the chloroplast and the cytoplasm of algal cells. In fact, isozymes of PGK have been detected in extracts of *Pisum sativum* (Anderson and Advani, 1970) and barley (MacMorrow and Bradbeer, 1987) leaves. If an antibody to PGK was to be used in studying the multi-enzyme complexes in the reductive pentose phosphate pathway (RPPP) it would be necessary to purify the isozyme localised in the chloroplast and raise an antibody against it.

Numerous attempts to separate PGK isozymes by gel filtration, ion exchange chromatography and SDS-polyacrylamide gel electrophoresis (PAGE), from both heterotrophic and autotrophic algal cells were unsuccessful. When a PGK preparation from autotrophically grown cells, which had previously been purified on DE-52™, Ultrogel AcA-44™ and FPLC mono Q™, was subjected to hydrophobic interaction chromatography on phenyl superose, a considerable spread of PGK activity was observed (Fig. V.1). Initially, the trailing fractions were thought to be a different isozyme to the peak fractions. The trailing fractions were pooled, dialysed and re-applied to the FPLC phenyl superose column. The spread in enzyme activity re-appeared (Fig. V.2), implying that hydrophobic interaction had not separated

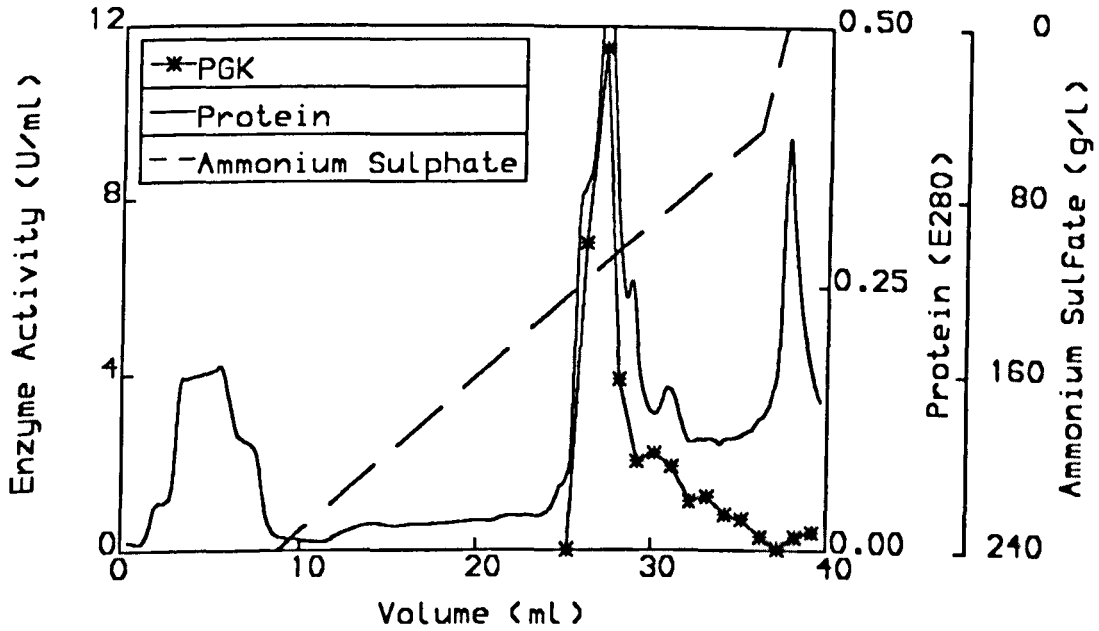


Figure V.1 Hydrophobic interaction chromatography of PGK on FPLC phenyl superose.

The gradient was developed with a decreasing concentration of ammonium sulphate in a phosphate buffer of ionic strength 0.221 and pH 7.0 on a HR5/5 column with a flow rate of 0.5mℓ/min. The extract from autotrophically grown *S. obliquus* had previously been purified on Whatman DE-52, Ultrogel AcA-44 and FPLC mono Q. The buffer was supplemented with 0.5mℓ/ℓ mercaptoethanol and 7.7mM sodium azide.

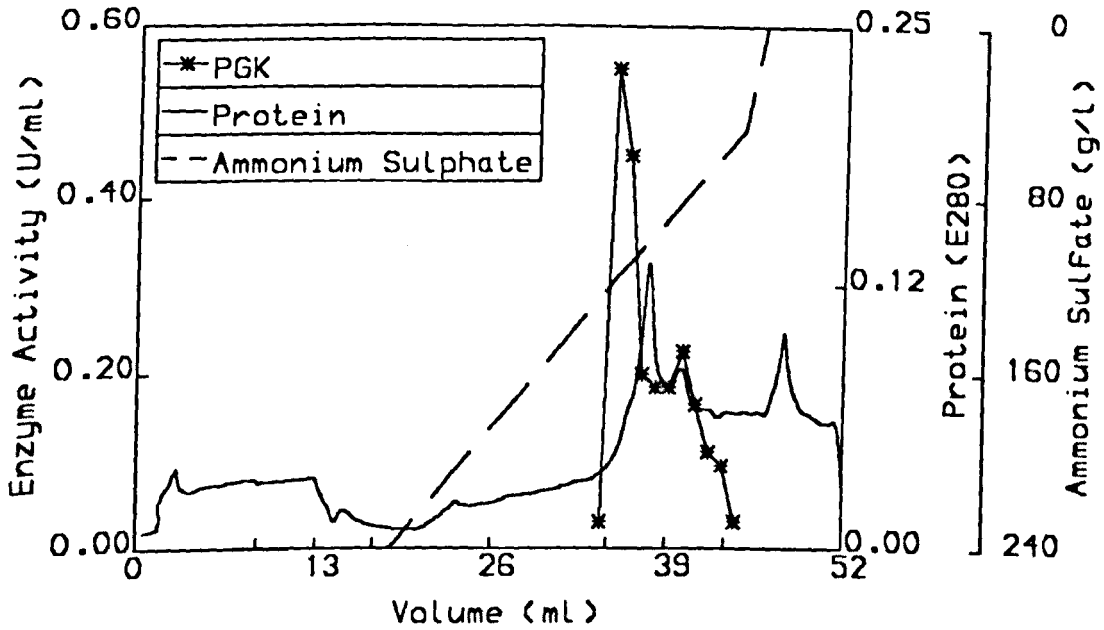


Figure V.2 Hydrophobic interaction chromatography of PGK on FPLC phenyl superose.

Trailing fractions of PGK from the previous hydrophobic interaction chromatography (Fig. V.1) were pooled, dialysed, concentrated and reapplied to FPLC phenyl superose. The gradient was developed with a decreasing concentration of ammonium sulphate in a phosphate buffer of ionic strength 0.221 on a HR5/5 column with a flow rate of 0.5mℓ/min. The buffer was supplemented with 0.5mℓ/ℓ mercaptoethanol and 7.7mM sodium azide.

PGK into isozymes. Thus if more than one isozyme existed in *Scenedesmus obliquus*, then they were indistinguishable by the techniques employed.

Having failed to detect a specific chloroplast PGK, the total cellular PGK from autotrophically grown cells was purified, and an antibody raised against it.

Choice of Buffers

During the purification of PGK, it was noticed that the levels of enzyme activity were considerably lower when phosphate buffers were used than with Tris-HCl buffers. Soluble proteins were extracted from the alga using either a Tris-HCl buffer of ionic strength 0.025 and pH 8.5 or a phosphate buffer of ionic strength 0.130 and pH 7.5, followed by chromatographing the crude extracts separately on Whatman DE-52™ using the relevant buffers. The gradients for protein elution were developed with Tris-HCl buffers of ionic strength 0.025 and 0.392 and pH 8.5 or phosphate buffers of ionic strength 0.028 (pH 8.0) and 0.392 (pH 7.7). The ratio of PGK to aldolase activities was much lower when the purification used phosphate buffers (Table V.1).

Step	Ratio of Activity of PGK to Aldolase	
	Phosphate	Tris-HCl
Crude Extract	0.22	1.50
DE-52 Eluant	0.40	2.00

Table V.1 The effect of Tris-HCl and phosphate buffers on the ratio of activities of PGK to aldolase.

When a sample of aldolase in a phosphate buffer of ionic strength 0.028 and pH 8.0 was redialysed against a Tris-HCl buffer of ionic strength 0.025 and pH 8.5, or *vice versa*, there was no significant change in enzyme activity. However, there was a considerable reduction in activity when PGK was transferred from the Tris-HCl buffer to the phosphate buffer. A change from the phosphate to the Tris-HCl buffers did not result in a significant increase in PGK activity. The various Tris-HCl buffers used were of comparable ionic strength to the corresponding phosphate buffers. The low levels of PGK activity when phosphate buffers were used probably resulted from an inactivation by phosphate. Consequently, purification of PGK was carried out using Tris-HCl buffers.

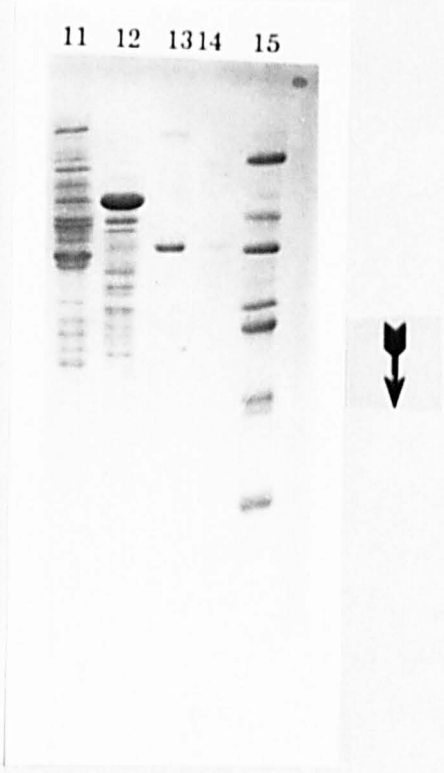
Initial Purification

Initial attempts to purify PGK consisted of ion exchange chromatography on Whatman DE-52, followed by gel filtration on Ultrogel AcA-44. SDS-PAGE of the peak fraction eluted from Ultrogel AcA-44 (Fig. V.3) indicated that there were many contaminating protein bands present. The PGK band was just below the major peptide band. Neither further ion exchange on an FPLC mono Q column nor a second mono Q step, using a shallower gradient, was able to remove all of the contaminating protein (Fig. V.4i).

Dye-ligand chromatography was then tested for its ability to further purify PGK, as PGK had previously been shown to bind to Cibacron™ blue F3GA™ (Kawai *et al.*, 1982). PGK eluted from gel filtration was found to bind to a Procion™ HE-7B™ dye-ligand column in the presence of a Tris-HCl buffer of ionic strength 0.025 and pH 8.5, and could be eluted with one of its substrates, 3-phosphoglycerate (PGA) (12mM in the Tris-HCl buffer). Surprisingly, PGA did not selectively elute PGK from this dye column, as could be seen by examining the eluted fractions with SDS-PAGE (Fig. V.4ii). Not all the PGK was eluted with PGA, some enzyme remained bound to the column in the presence of PGA and this was eluted with 2M KCl. However, elution with PGA turned out to be very inconsistent as PGA often did not elute PGK activity. ATP, another substrate of PGK was substituted for PGA. This nucleotide, at a concentration of 6mM also eluted PGK activity from the HE-7B column. Moreover, this elution

Figure V.3 SDS-PAGE of PGK eluted from Ultrogel AcA-44.

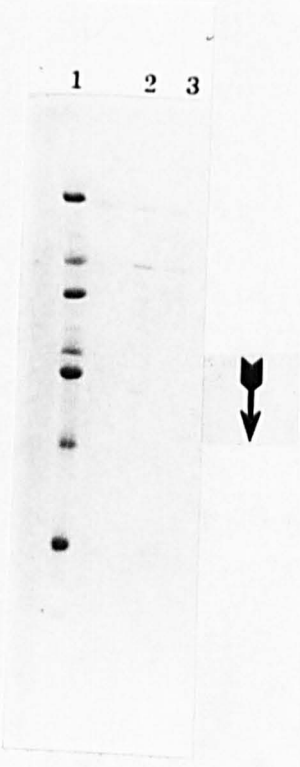
Extract from autotrophically grown *S. obliquus* had previously been purified on Whatman DE-52. [11 and 12] peak fractions of PGK from two different gel filtration experiments; [15] M_r marker proteins (66 000, 45 000, 36 000, 29 000, 24 000, 20 000 and 14 200).



PHOSPHOGLYCERATE KINASE

Figure V.4i SDS-PAGE of PGK eluted from FPLC mono Q.

Extract from autotrophically grown *S. obliquus* had previously been purified on Whatman DE-52 and Ultrogel AcA-44. [1] M_r marker proteins (66 000, 45 000, 36 000, 29 000, 24 000, 20 000 and 14 200); [2] eluted from FPLC mono Q. [3] protein sample depicted in track 2 was dialysed, concentrated and re-applied to FPLC mono Q and eluted with a shallower phosphate gradient.



PHOSPHOGLYCERATE KINASE

Figure V.4ii SDS-PAGE of PGK eluted from Procion red HE-7B and FPLC mono Q.

Extract from autotrophically grown *S. obliquus* had previously been purified on Whatman DE-52 and Ultrogel AcA-44. [9] Protein that did not bind to the column in the presence of Tris-HCl buffer of ionic strength 0.025 and pH 8.5; [10] protein eluted with 12mM PGA in the same buffer; [13] M_r marker proteins (66 000, 45 000, 36 000, 29 000, 24 000, 20 000 and 14 200). The buffer was supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

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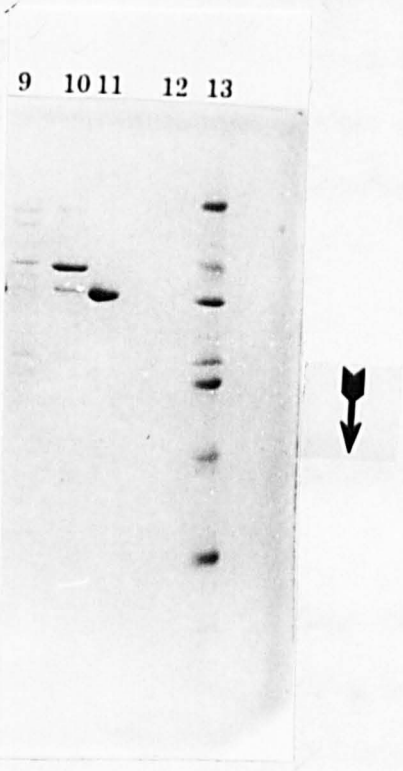


PHOSPHOGLYCERATE KINASE

was reproducible. Unfortunately, ATP like PGA, eluted other proteins along with PGK, these could not be removed by further chromatography on FPLC mono Q (Fig. V.5).

Substituting hydrophobic interaction chromatography for dye-ligand chromatography in the purification procedure, proved to be successful. Consequently a method involving ion exchange chromatography, gel filtration and hydrophobic interaction chromatography was used to isolate PGK from *S. obliquus*.

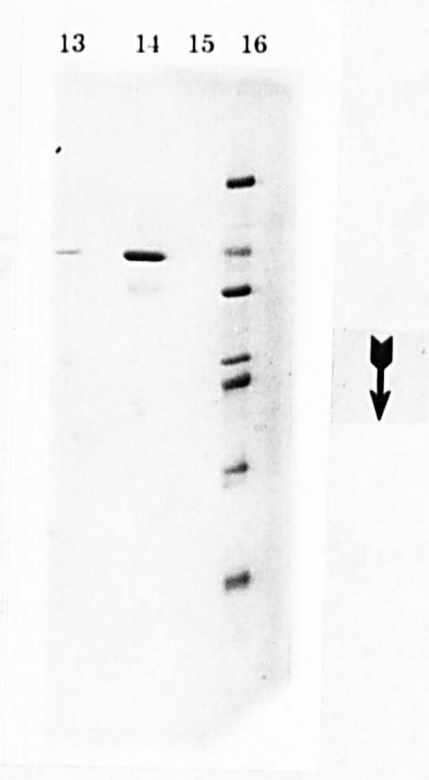
Purification of PGK

A batch of autotrophically grown *S. obliquus* cells was divided into six portions and each portion was ground with glass beads. The crude extract was divided into four equal portions and each portion was applied to a Whatman DE-52 column (Fig. V.6). PGK was one of the first proteins to elute from the column following the application of the Tris-HCl gradient. The fractions with the highest PGK activity eluting from all four columns were pooled, concentrated and divided into three portions.

Each portion was chromatographed separately on an Ultrogel AcA-44 gel filtration column (Fig. V.7). The characteristic spread of enzyme activity on gel filtration was apparent. The best fractions from all three chromatographic runs were pooled, concentrated and divided

Figure V.5 SDS-PAGE of PGK eluted from Procion red HE-7B and FPLC mono Q.

Extract from autotrophically grown *S. obliquus* that had previously been purified on Whatman DE-52 and Ultrogel AcA-44, was applied to Procion red HE-7B. [14] protein eluted from Procion red HE-7B with 10mM ATP; [13] ATP-eluted protein from Procion red HE-7B (track 14) was applied to FPLC mono Q and eluted with a Tris-HCl gradient made by mixing buffers of ionic strength 0.025 and 0.392 and pH 8.5; [16] M_r marker proteins (66 000, 45 000, 36 000, 29 000, 24 000, 20 000 and 14 200).



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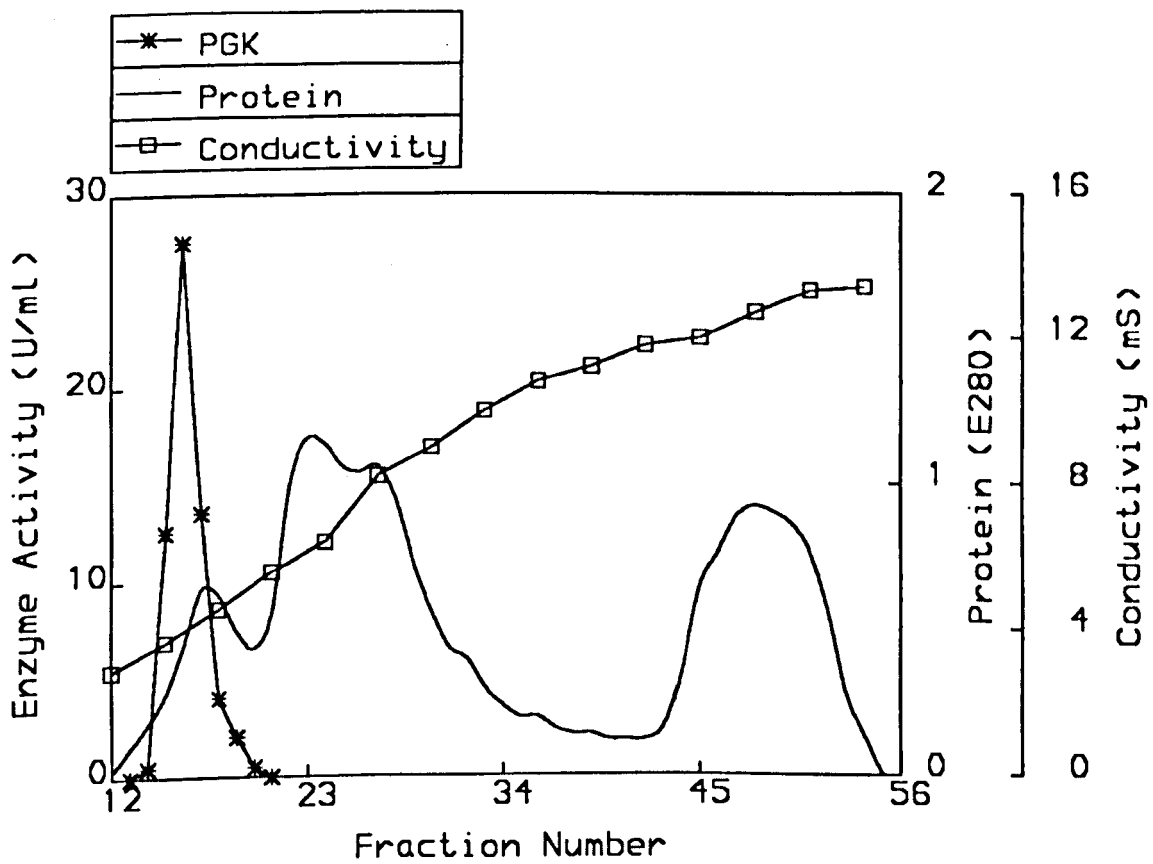

Figure V.6 Ion exchange chromatography of crude extract from autotrophically grown S. obliquus on Whatman DE-52.

The gradient was developed by mixing Tris-HCl buffers of ionic strength 0.025 and 0.392 and pH 8.5 on a 2.2 × 29.5cm column with a flow rate of 50mℓ/h. Fractions of 6mℓ were collected. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

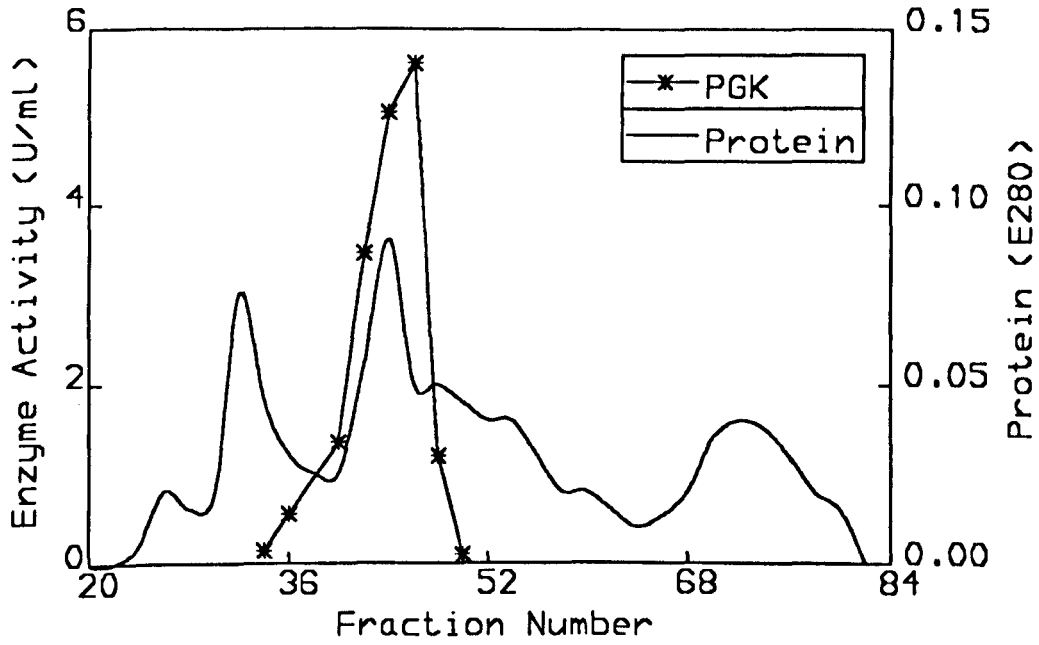

Figure V.7 Gel filtration of PGK on Ultrogel AcA-34.

Elution was performed on a 2.6 × 83.0cm column at pH 8.5 with a flow rate of 20mℓ/h. The extract from autotrophically grown *S. obliquus* had previously been purified on Whatman DE-52. The eluting buffer was Tris-HCl with an ionic strength of 0.025 and supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol. Fractions of 3mℓ were collected.

into six portions. Each portion was subjected to ion exchange chromatography on the FPLC mono Q column (Fig. V.8). Although the major protein peak eluted in the fractions possessed PGK activity, SDS-PAGE detected the presence of other proteins in these fractions (Fig. V.9). The best fractions from all four runs were pooled, concentrated and divided into six portions.

Each portion was subjected to hydrophobic interaction chromatography on a FPLC phenyl superose column (Fig. V.10). Under hydrophilic conditions (high concentration of ammonium sulphate), proteins adsorbed through their hydrophobic domains to the phenyl superose. These adsorbed proteins, including PGK, were differentially desorbed by eluting with a decreasing gradient of ammonium sulphate (*i.e.* increasing hydrophobicity). The single protein peak corresponding to PGK activity during ion exchange on FPLC mono Q was resolved into two protein peaks during hydrophobic interaction chromatography. It was the smaller protein peak eluted earlier, which corresponded to the PGK activity peak. The fractions with the highest activity of PGK from all six runs were pooled. Considerable trailing of enzyme activity was readily apparent.

The protein concentration of the best fractions pooled from the former hydrophobic interaction step was too low to be detectable on SDS-PAGE using the Coomassie-blue stain. Since silver staining was a much more sensitive technique for visualising proteins in polyacrylamide gels (Switzer *et al.*, 1979), the method of Wray *et al.* (1981) was attempted, whic did detect the presence of only a single

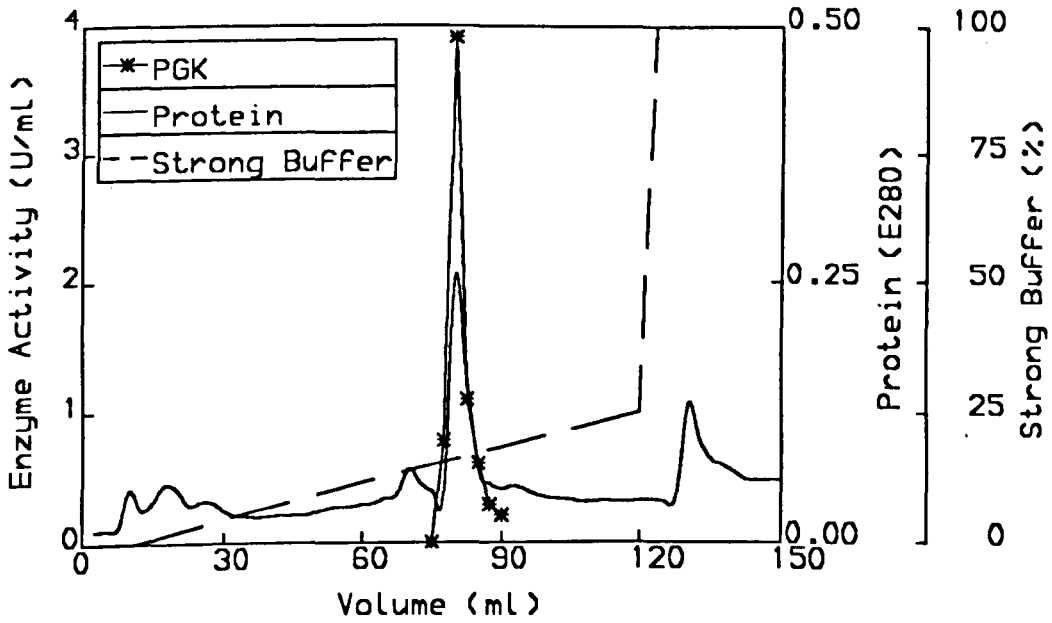

Figure V.8 Ion exchange chromatography of PGK on FPLC mono Q. The gradient was developed by mixing Tris-HCl buffers of ionic strength 0.025 and 0.392 and pH 8.5 on a HR10/10 column with a flow rate of 4mℓ/min. Buffers were supplemented with 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol.

Figure V.9 SDS-PAGE of PGK eluted from FPLC mono Q.

Extract from autotrophically grown *S. obliquus* had previously been purified on Whatman DE-52 and Ultrogel AcA-44. [6 and 7] peak fractions of PGK from different FPLC mono Q experiments; [10] M_r marker proteins (66 000, 45 000, 36 000, 29 000, 24 000, 20 000 and 14 200).


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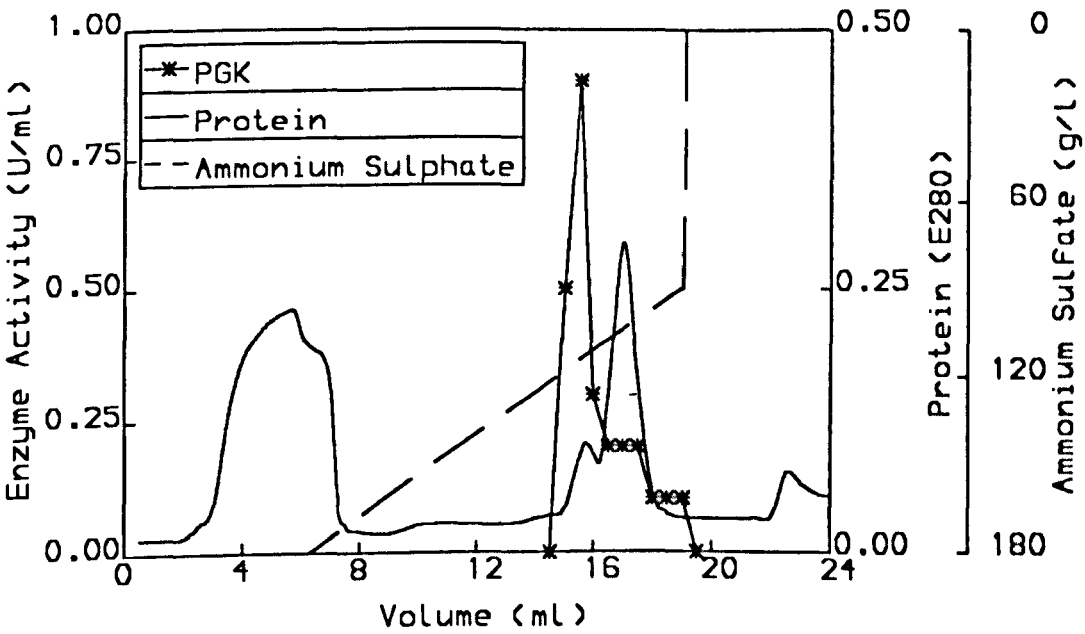

Figure V.10 Hydrophobic interaction chromatography of PGK on FPLC phenyl superose.

The gradient was developed with a decreasing concentration of ammonium sulphate in a phosphate buffer of ionic strength 0.221 and pH 7.0 on a HR5/5 column with a flow rate of 0.5mℓ/min. The extract from autotrophically grown *S. obliquus* had previously been purified on Whatman DE-52, Ultrogel AcA-44 and FPLC mono Q. The buffer was supplemented with 0.5mℓ/ℓ mercaptoethanol and 7.7mM sodium azide.

protein band. Unfortunately, the staining reaction could not be stopped at the appropriate time, leading to the PGK protein band being overshadowed by the background.

The details of the purification are shown in Table V.2. The purification was accompanied by a very poor yield. Scopes (1969) attributed the low levels of PGK recovery to inhibition by heavy metal impurities. This low yield was especially apparent during FPLC on the mono Q and phenyl superose columns. This purification protocol produced purified PGK, from *S. obliquus*, on an analytical scale, although the quantity of enzyme obtained was sufficient to raise an antibody in a Dutch rabbit.

Step	Total Volume (mℓ)	Protein Conc. (mg/mℓ)	Enzyme Activity (U/mℓ)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Crude extract	2 441	15.23	2.65	0.17	1.0	100.0
DE-52	1 670	6.60	13.05	1.98	11.4	68.5
AcA-54	915	0.44	3.00	6.82	39.2	37.5
mono Q	200	0.06	1.68	27.92	160.4	8.4
phenyl superose	31	0.03	1.74	58.00	333.3	1.3

Table V.2 Purification profile of PGK extracted from autotrophically grown *S. obliquus*.

Raising of Antiserum

The purified PGK preparation was injected into the rabbit in two doses. The antibody raised was shown to bind to PGK, but

unfortunately there was a double band in the western blotting of SDS-PAGE of both crude algal extract and purified PGK from *S. obliquus* (Fig. V.11). The first and second antibodies raised were stored at -20°C, until required for further use.

Properties

The molecular weight of the subunit of PGK, estimated by SDS-PAGE to be 45 000 (Fig. V.12), compared favourably with the size of 43 500 obtained for halibut muscle PGK (Huskins *et al.*, 1982). Gel filtration of the native enzyme on Ultrogel AcA-44 yielded a molecular weight of 38 000 (Fig. V.13), which was over 15% less than the size of the subunit. Andrews (1965) estimated that the level of uncertainty in the determination of the molecular weight of globular proteins using gel filtration was 10%. Nevertheless, it was a rapid and simple method for obtaining approximate values whilst purifying proteins. These two results together would suggest that PGK isolated from *S. obliquus* was a monomeric enzyme, in agreement with the assertion by Banks *et al.* (1979) that PGK isolated from a wide variety of sources was monomeric.

Figure V.11 Western blot using antiserum raised against PGK.

Protein samples were subjected to SDS-PAGE prior to blotting. [1] crude extract from heterotrophically grown *S. obliquus*; [2] crude extract from autotrophically grown *S. obliquus*; [3] purified PGK.

PHOSPHOGLYCERATE KINASE


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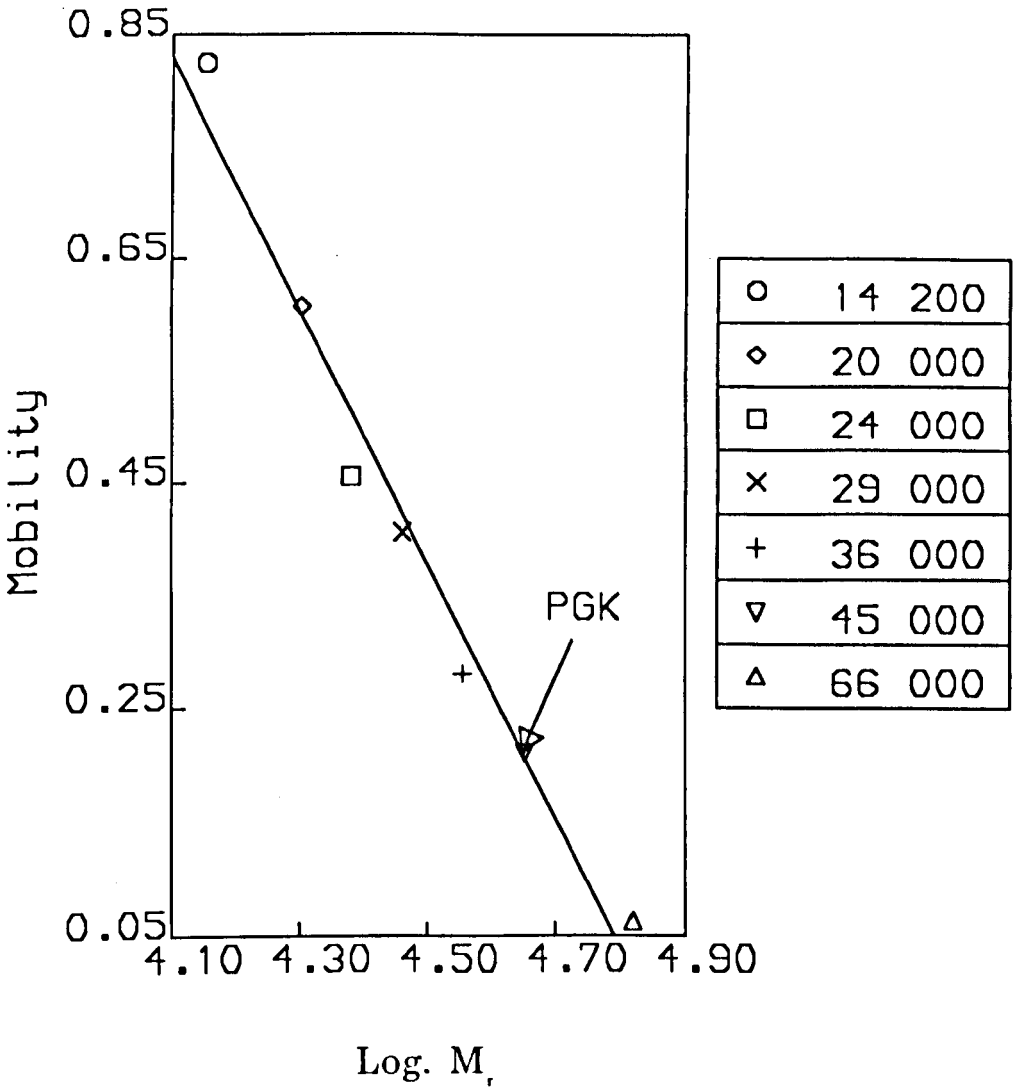

Figure V.12 M_r determination of the PGK subunit by SDS-PAGE. The mobilities were measured from the gel and the plot was used to obtain their M_rs.

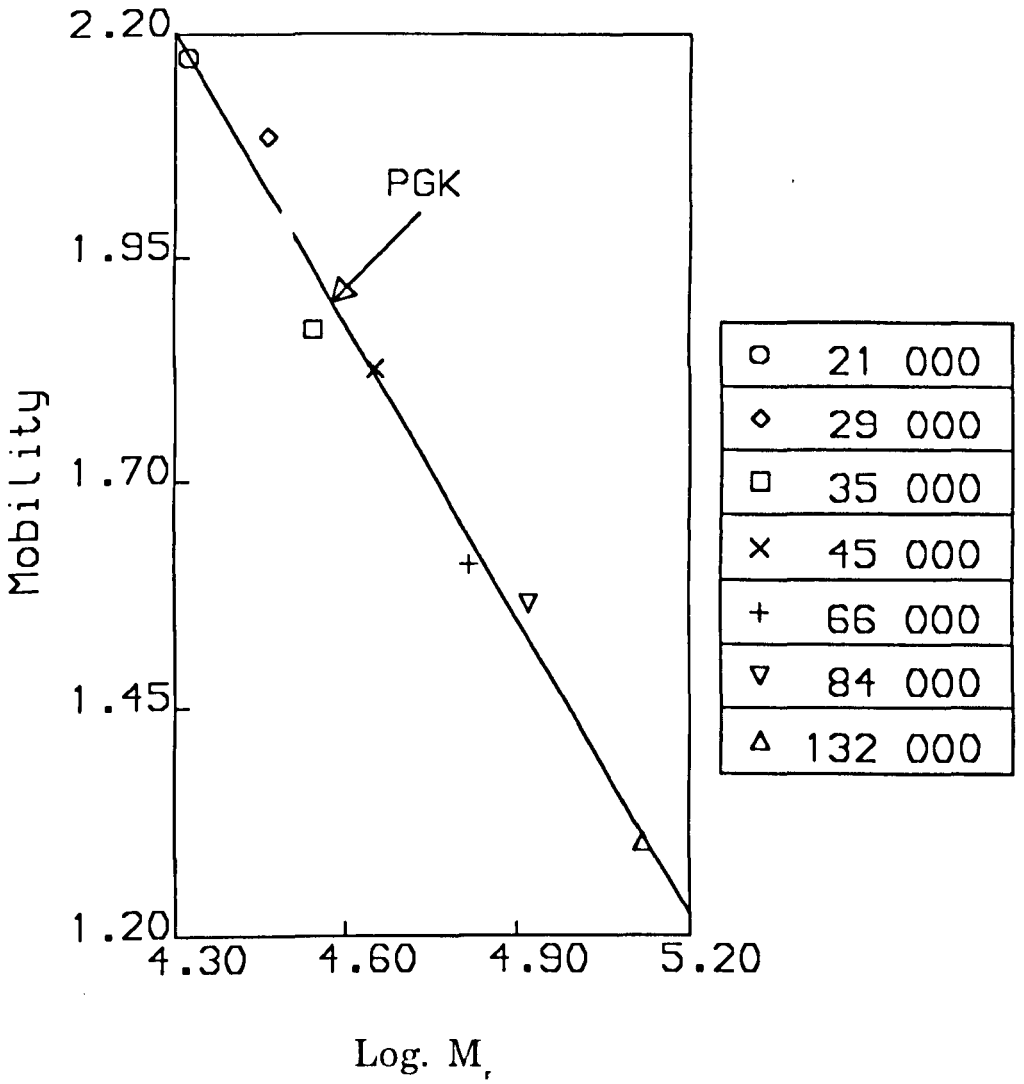

Figure V.13 M_r determination of native PGK by gel filtration.

The mobilities were measured from the Ultrogel AcA-44™ elution profile and the above plot was used to obtain their M_rs.

Complexed PGK

An attempt was made to detect the presence of multi-enzyme complexes in the crude extract of *S. obliquus*. If the intramolecular forces binding enzymes together in a complex were too weak to withstand chromatography on Whatman DE-52, one would have expected the enzymes to elute separately from Whatman DE-52. Although RuBPC co-eluted with PRK (Fig. III.1), the other three RPPP enzymes studied eluted separately. Since gel filtration was considered to be a gentler technique, and especially since any complex would have been of a high molecular weight, the crude extract was directly applied to the Ultrogel AcA-34™ column.

Initially, no evidence for the presence of complexes was obtained. The breaking and equilibrationg buffers used were then changed from phosphate to Tris-HCl. Various supplements were then added to the buffers in an attempt to stabilise any complex(es) *in vitro*. After supplementing the buffers with 250mM sucrose and 5mM magnesium ions, in an attempt to stabilise cell membrane-protein interactions (Hoober, 1984), PGK activity was detected in the void volume of the Ultrogel AcA-34 column (Fig. V.14). Because of its small size, any uncomplexed PGK would have eluted much later. This finding suggested that PGK was probably bound to another molecule or molecules, with the resulting complex having a considerably higher molecular weight. The PGK activity of the enzyme form with high

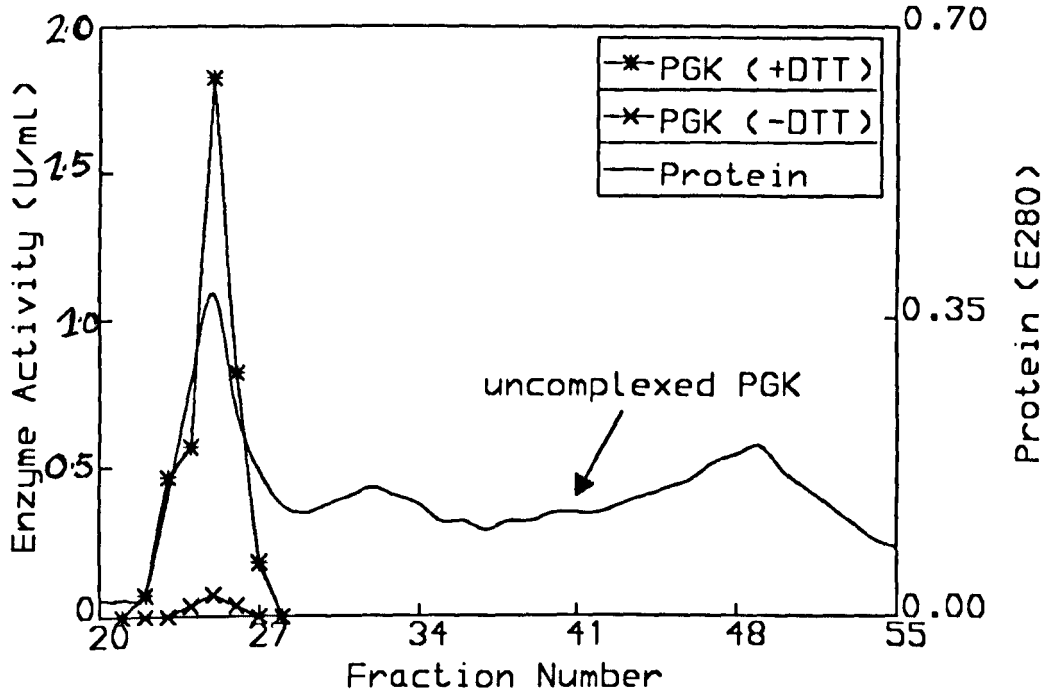

Figure V.14 Gel filtration of crude extract from *S. obliquus* on Ultrogel AcA-34.

Elution was performed on a 2.6 × 83.0cm column at pH 8.5 with a flow rate of 20mℓ/h. The eluting buffer was Tris-HCl with an ionic strength of 0.025 and supplemented with 250mM sucrose, 5mM magnesium ions, 0.5mℓ/ℓ mercaptoethanol, 7.7mM sodium azide and 10% v/v glycerol. Fractions of 3mℓ were collected.

molecular weight was greatly enhanced by incubation with DTT, prior to assaying.

Unfortunately, attempts to reproduce this result proved fruitless. None of the other four RPPP enzyme activities were ever detected in or near the void volume during gel filtration on AcA-34.

DISCUSSION

Since the enzymes of the RPPP mediate reactions which constitute consecutive steps in a metabolic pathway, the existence of enzymes physically linked together for facilitating the sequential transfer of substrate/product molecules is a distinct possibility (Akazawa, 1970). Recent reports suggest the occurrence of associations between certain enzymes of this cycle in *Pisum sativum* (Sainis and Harris, 1986), *Spinacia oleracea* (Gontero *et al.*, 1988), and *Scenedesmus obliquus* (Nicholson *et al.*, 1987).

The complex isolated from *S. obliquus* consisted of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and phosphoribulokinase (PRK). Since these two enzymes did not catalyse consecutive reactions and were activated upon dissociation, the possible role envisaged for their complexing was that of regulation. The multi-enzyme complex in *S. oleracea* was reported by Gontero *et al.* (1988) to consist of G3PDH, PRK, phosphoriboisomerase, ribulose 1,5-bisphosphate carboxylase, and phosphoglycerate kinase (PGK). The occurrence of both, G3PDH and PRK in this complex, suggests that the G3PDH-PRK complex in *S. obliquus* might be a constituent of a larger multi-enzyme complex *in vivo*.

A surprising feature of the *S. oleracea* multi-enzyme complex was its dependence on NADH, which is in variance with the fact that NADPH is the nucleotide utilised by the RPPP. Although this feature was also

characteristic of the *S. obliquus* multi-enzyme complex, this discrepancy was eliminated once the complex was activated.

Concrete evidence for the presence of other protein-protein interactions between the enzymes of the RPPP of *Scenedesmus obliquus* has not been forthcoming. Loose associations of enzymes form unstable complexes (Fulton, 1982; Clegg, 1984), which may be disrupted upon extraction (Gaertner, 1978) and dilution (Clarke and Masters, 1974). Evidence suggesting that phosphoglycerate kinase (PGK) was probably bound to (an)other molecule(s) was not reproducible. Nevertheless it is still possible that PGK could be involved in loose protein-protein interactions, which due particularly to the harsh method employed to break the cell walls, might be liable to disruption.

PGK activity of high molecular weight was greatly enhanced by incubation with DTT prior to assay. PGK could have been inactive whilst complexed, but this would have meant that the complexing of PGK was of no benefit to the alga. It was more likely that the enzyme was not accessible to its substrate, supplied exogenously, whilst in the complex. Possibly PGA has to be channelled to the complexed PGK from ribulose 1,5-bisphosphate carboxylase (RuBPC), which therefore would have been part of the complex. DTT may have caused the dissociation of the complex, thus making PGK accessible to the PGA supplied in the assay. DTT had previously been shown by Bradbeer *et al.* (1981) to mimic *in vitro* the *in vivo* activation mediated by photoreduced thioredoxin, probably by reducing disulphide bonds (Anderson and Avron, 1976).

Whilst purifying fructose 1,6-bisphosphate (FBP) aldolase, multiple forms were detected. Two forms were expected, one localised in the chloroplast and the other in the cytoplasm, but surprisingly three were encountered. Due to the extremely tough cell wall of *S. obliquus.*, attempts to isolate intact chloroplasts, proved to be fruitless. Hence, the cellular localisation of these isozymes had to be determined using indirect means.

The definition of the term 'isozyme' was revised by the International Union of Pure and Applied Chemistry-International Union of Biochemistry in 1976 and now requires a difference in primary structure (IUPAC-IUB, 1976). Since recent relevant publications refer to the different forms of aldolase present in algal cells as isozymes, the multiple forms occurring in *S. obliquus* were also referred to as such.

All three isozymes from *S. obliquus* had similar properties and studies of the isozymes from leaves of *S. oleracea* (Lebherz *et al.*, 1984) and *P. sativum* (Anderson and Pacold, 1972) also indicated that these species possessed very similar isozymes. Although, there seems to be no advantage for the plant to have exceedingly similar isozymes, there is no reason to believe that the chloroplast isozyme is more effective in synthesising FBP, and the cytoplasmic isozyme more suitable for cleaving FBP (Anderson and Pacold, 1972).

Since the antibody raised against G3PDH was not specific enough, attempts are being made in this laboratory to raise a new antibody. The specificity of the antisera against RuBPC, PRK and aldolase are

adequate to be used in the detection of multi-enzyme complexes in crude cell extracts. It would also be of interest to test for cross-reactions between the *S. obliquus* antibodies and the enzymes of the RPPP or glycolysis from other species, especially higher plants and other green algae.

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