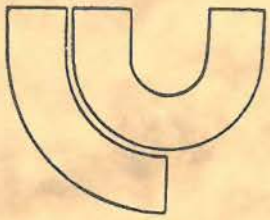

Limburgs Universitair Centrum
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Purification, molecular cloning and expression of the cDNA of bovine pregastric esterase

Zuivering, clonering en expressie van het cDNA van het bovien pregastrisch esterase

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Medische Wetenschappen
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Promotoren: Prof. Dr. H. Teuchy
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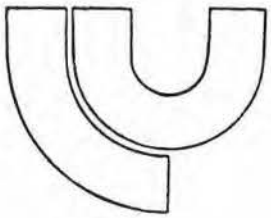

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Prof. Dr. G. Volckaert, KUL

Prof. Dr. L. Wijns, VUB

Dr. L. Kupers, LUC

Prof. Dr. H. Teuchy, LUC

List of Abbreviations

AOX	Alcohol oxidase
AP	Alkaline phosphatase
APS	Ammoniumpersulfate
BCA	Bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bp	Base pairs
BSA	Bovine serum albumin
$C_{12:0}$	Lauric acid
cDNA	Complementary DNA
CIP	Calf intestinal phosphatase
CMC	Critical micellar concentration
$C_{12:0}$-TNB	Dodecyldithio-5-(2-nitrobenzoic acid)
Da	Dalton
DAB	3,3'-Diaminobenzidine
DEPC	Diethylpyrocarbonate
DGL	Dog gastric lipase
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleoside triphosphate
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
E600	Diethyl p-nitrophenyl phosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GST	Glutathione-S-transferase
HDL	High density lipoprotein
HGL	Human gastric lipase
HL	Hepatic lipase
HRP	Horse radish peroxidase
IDL	Intermediate density lipoprotein
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobase pairs
kDa	Kilodalton
LB	Luria broth
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
mAb(s)	Monoclonal antibody(ies)

MOPS	3-(N-morpholino)propanesulfonic acid
NBT	Nitro blue tetrazolium
OD	Optical density
OPD	o-Phenylenediamine
pAb(s)	Polyclonal antibody(ies)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
4-PDS	4,4'-Dithiopyridine
PEG	polyethylene glycol
pfu(s)	Plaque forming unit(s)
PGE	Pregastric esterase
PL	Pancreatic lipase
PNPB	p-Nitrophenyl butyrate
PVDF	Polyvinylidene difluoride
RGL	Rabbit gastric lipase
RLL	Rat lingual lipase
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulphate
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TEMED	N,N,N,N,-tetramethylethylene diamine
THL	Tetrahydrolipstatin
TPCK	1-Tosylamide-2-phenylethyl chloromethyl ketone.
Tris	Tris(hydroxymethyl)aminomethane
U	Unit(s)
VLDL	Very low density lipoprotein
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. Lipases: An overview

1.1 Introduction

1.1.1 Lipases in general

Lipases are triacylglycerol acylhydrolases (EC 3.1.1.3). They are widely distributed in animals, plants and micro-organisms. Lipases have been defined as specialised esterases with the ability of hydrolysing substrates insoluble in water. This sets them apart from the true esterases which are only active on substrates soluble in water.

1.1.2 Alimentary triacylglycerols and their metabolism

At birth the new-born mammal has to adapt immediately to a high milk intake which provides 70% of the energy as fat. In the adult about 35% of the daily energy requirements are met by fat. Dietary fat consists for 95% of triacylglycerols, the remaining part being made up by cholesterol and phospholipids.

Triacylglycerols are neutral lipids which are not soluble in water but instead form emulsions in it. Their structure is shown in fig. 1-1.

Triacylglycerols from animals or plants contain mainly long chain fatty acids ($\geq$ 14 carbon atoms). Short chain fatty acids ($\leq$ 6 carbon atoms) are less frequent and are almost exclusively found in milk triacylglycerols, especially cow's milk. They are preferentially situated at the *sn*-3 position.

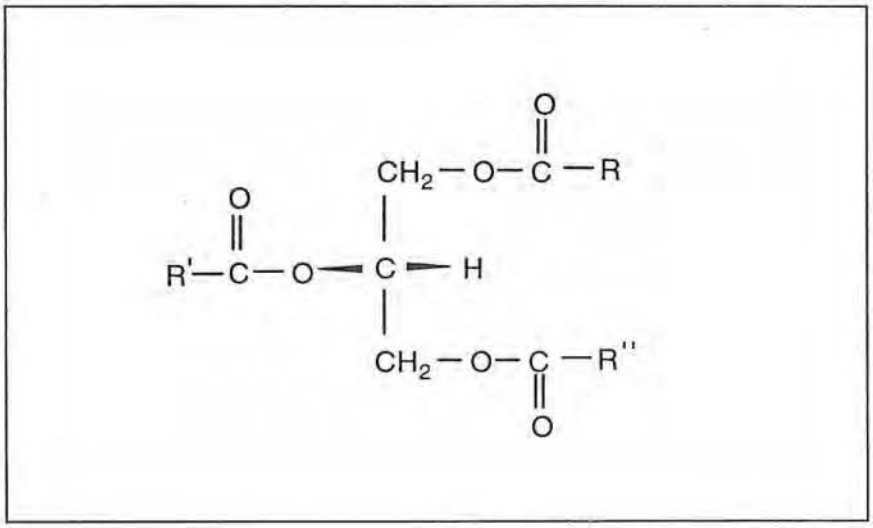

Fig. 1-1 Stereochemical configuration of triacylglycerols

Between the moment of uptake until their usage or storage, triacylglycerols undergo several cycles of hydrolysis and resynthesis. Different lipolytic enzymes intervene in this process. An overview of the steps involved in the metabolism of alimentary triacylglycerols is summarised in fig. 1-2.

In mammals three groups of lipolytic enzymes can be distinguished: digestive lipases discharged into the digestive tract (preduodenal, pancreatic and carboxyl ester lipases), tissue lipases (lysosomal, lipoprotein, hepatic and hormone sensitive lipase) and milk lipase or bile salt stimulated lipase. Based on similarity of characteristics and primary sequence identity these lipolytic enzymes can also be classified in different lipase families (Table 1-1).

Before dietary triacylglycerols are taken up and used by the body, they must be broken down in the lumen of the gastro-intestinal tract. The digestive lipases hydrolyse the triacylglycerols into free fatty acids and 2-mono-acylglycerols. These reaction products are absorbed by the epithelial cells of the small intestine. Upon their entry into the cells, long chain fatty acids first attach to a fatty acid binding protein. After activation into their acyl-CoA derivatives, the fatty acids are reesterified with the 2-monoacylglycerols into triacylglycerols. After resynthesis,

the triacylglycerols form lipoprotein complexes (chylomicrons) with phospholipids and apolipoproteins. These complexes are released from the cells into the lymphatics. The intestinal lymph vessels finally drain into the venous circulation via the thoracic duct. The chylomicrons are distributed by the blood through the whole organism. Chylomicrons are the main vehicle of transport of dietary long chain fatty acids. Short and medium chain fatty acids ($\leq$ 12 carbon atoms) are directly passed by the intestinal cells into the portal blood and metabolised in the liver.

Lipoprotein lipase and hepatic lipase are responsible for the hydrolysis of the triacylglycerols in the circulating lipoproteins. The reaction products that are absorbed by the cells are either used as fuel or resynthesized for storage. Intracellular triacylglycerols which are stored in the adipocytes can be released by the action of hormone sensitive lipase and monoacylglycerol lipase.

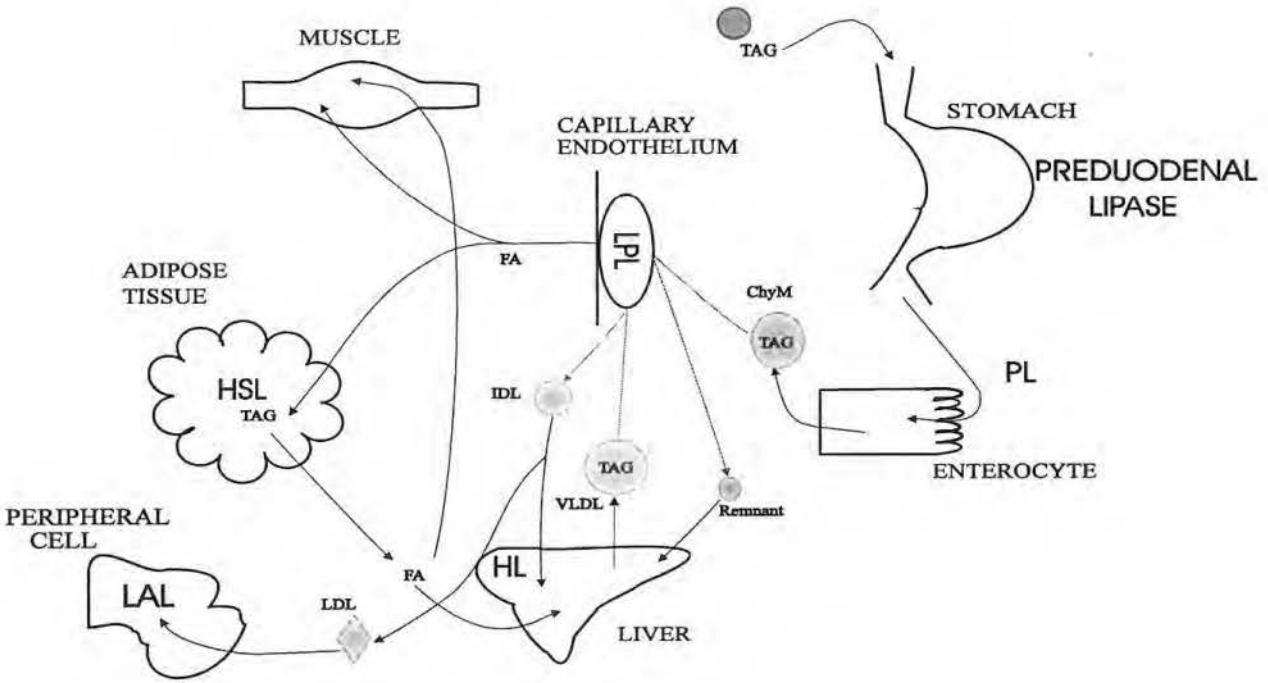

Fig. 1-2 Overview of the steps in alimentary triacylglycerol metabolism

ChyM, chylomicron; FA, fatty acid; HL, hepatic lipase: HSL, hormone sensitive lipase; IDL, intermediate density lipoprotein; LAL, lysosomal acid lipase; LDL, low density lipoprotein; LPL, lipoprotein lipase; PL, pancreatic lipase; TAG, triacylglycerol; VLDL, very low density lipoprotein

Table 1-1 Overview of mammalian lipase families and their characteristics

Lipase family	Type of lipase	Site of production	Site of action	Substrates	pH optimum	Cofactor	References
Acid lipases	Preduodenal lipase	Tongue, pharynx, stomach	Stomach	Triacylglycerols	4-5	none	Gargouri et al., 1989
	Lysosomal lipase	Lysosomes	Lysosomes	Triacylglycerols Cholesterol esters	4.5-5	none	Fowler and Brown, 1984
Neutral lipases	Pancreatic lipase	Exocrine cells of the pancreas	Small intestine	Triacylglycerols	8-9	Colipase	Verger, 1984
	Lipoprotein lipase	Parenchymal cells of skeletal muscle, adipose tissue, lung, kidney, aorta, lactating mammary gland	Capillary lumen	Chylomicrons VLDL	8-8.5	Apo CII	Wang et al., 1992
	Hepatic lipase	Parenchymal cells of the liver	Capillary lumen	HDL IDL	8-8.5	none	Kinnunen, 1984
Bile salt activated lipases	Carboxyl ester lipase	Exocrine cells of the pancreas	Small intestine	Triacylglycerols Phospholipids Cholesterol esters	8	Bile salts	Wang and Hartsuck, 1993
	Bile salt stimulated lipase	Lactating mammary gland	Small intestine	Triacylglycerols Phospholipids Cholesterol esters	8	Bile salts	Wang and Hartsuck, 1993
	Hormone sensitive lipase	Adipose tissue	Adipocytes	Triacylglycerols	7	none	Belfrage et al., 1984

1.2 Acid lipase family

1.2.1 Preduodenal lipases

Dietary lipids must be hydrolysed by lipases before their absorption in the intestine. Until quite recently the digestion of fats was almost exclusively attributed to the activity of pancreatic lipase in the duodenum.

As early as 1901 Volhard showed that human gastric juice could hydrolyse triacylglycerols. He concluded that the stomach was an important site for fat digestion. For a long time however this gastric lipolysis was considered to be the result of a duodenal reflux of pancreatic juices.

The enzymes responsible for gastric lipolysis are known by the general name of preduodenal lipases (Gargouri et al., 1989; Hamosh 1990). According to their main site of secretion they are called lingual lipases (secreted by von Ebner's glands in the tongue), pharyngeal or gastric lipases. The mouse and the rat have a lingual lipase. Polygastric species like cow and sheep have a pharyngeal lipase. Rabbit, dog and man have a gastric lipase (Moreau et al., 1988).

1.2.1.1 Calf pregastric esterase

The pharyngeal lipase of the calf is also known as pregastric esterase (PGE). It is the major fat digestive enzyme in the young animal. During the period of milk intake the digestive system of the ruminant resembles that of monogastric (abomasum) species. In the abomasum milk is clotted by the action of rennin (chymosin) and pepsin. This prevents the rapid passage of milk from the stomach and allows a slow digestion. Triacylglycerol hydrolysis in the abomasum is a result of the action of PGE. The enzyme has a specificity for short and medium chain fatty acids. Furthermore it preferentially attacks the *sn*-3 position, where the short chain fatty acids are mainly located in milk fat triacylglycerols. The amount of PGE secreted is not influenced by diet or age (Young et al., 1960). However, the manner in which the animals are fed appears to be important. Suckling provides a great stimulus for PGE secretion (Wise et al., 1976). In the young ruminant PGE is responsible for 65-70% of the hydrolysis of milk fat (Gooden and

Lascelles, 1973). The remainder is digested in the intestine by pancreatic lipase. Immediately after birth the secretion of pancreatic lipase and bile salts is very low, but within a few days the output increases considerably. As pancreatic lipase has a wider positional and fatty acid specificity than PGE, it is able to continue the process of lipid digestion.

After the diet switches from milk to solid food the polygastric arrangement (rumen, reticulum, omasum and abomasum) becomes functional. For the adult ruminant a complete population of micro-organisms is present in the rumen. These micro-organisms produce lipases that split triacylglycerols completely to glycerol and free fatty acids which are further fermented. Consequently, the lipolytic activities of PGE and of pancreatic lipase are probably less important in the adult animal.

1.2.1.2 The importance of gastric lipolysis.

The physiological contribution of gastric lipase to overall lipolysis of dietary fat in the normal human adult has been evaluated (Carrière et al., 1993). According to this study 10% of the fatty acids in dietary triacylglycerols are released in the stomach. Considering that hydrolysis of triacylglycerols into monoacylglycerols (= 66%) is sufficient for complete fat absorption, there remains 56 % to be hydrolysed in the duodenum. It has been calculated that 7.5 % of this duodenal lipolysis is due to the action of human gastric lipase (HGL). These data clearly show that, although pancreatic lipase is the main enzyme for dietary fat digestion in normal subjects, gastric lipase is certainly not a minor enzyme in the overall fat digestive process.

For adults with normal pancreatic and hepatic functions an initial digestion of fat in the stomach is required for efficient intestinal lipolysis (Gargouri et al., 1986). There are several reasons for this requirement. The release of fatty acids stimulates the secretion of cholecystokinin by epithelial endocrine cells of the small intestine, particularly the duodenum. This in turn stimulates the secretion of pancreatic lipase. Furthermore, partially digested triacylglycerols are better and faster hydrolysed by pancreatic lipase (Borel et al., 1994) while free fatty acids increase the binding of colipase to bile salts and to lipase (Patton et al., 1978).

Gastric lipolysis has a significant role in the digestion of fat in individuals suffering from pancreatic insufficiency of developmental or pathological origin.

Lipolysis by gastric lipases is very important in patients with pancreatitis due to chronic alcoholism (Abrams et al., 1987) or to cystic fibrosis (Abrams et al., 1984). In patients with pancreatic insufficiency the extent of lipolysis by preduodenal lipases may amount to as much as 50% of dietary fat (Muller et al., 1975; Fredrikzon and Bläckberg 1980). This is because in these subjects the activity of gastric lipase is better preserved in the small intestine. The lower pH in their duodenum and the absence of pancreatic proteolytic enzymes accounts for the fact that gastric lipase can continue its catalytic action for a longer period of time than in normal subjects. Therefore a substantial intestinal fat hydrolysis by gastric lipases occurs. There is no evidence for an increased production of gastric lipase to compensate for the decreased pancreatic lipase secretion in pancreatic insufficiency (Moreau et al., 1988).

In the newborn partial fat hydrolysis in the stomach is required for the digestion of fat globules in milk or infant formulas (Hernell and Bläckberg 1994). Newborns indeed produce only low amounts of pancreatic lipase and bile salts. Furthermore, it has been demonstrated that pancreatic lipase of itself does not readily hydrolyse native milk fat globules. After preincubation of the milk with preduodenal lipase however, the pancreatic lipase very efficiently hydrolyses milk fat droplets (Bernbäck et al., 1989). The combination of gastric and pancreatic lipase hydrolyses two thirds of the ester bonds in these droplets. Total digestion requires the action of bile-salt-activated lipase. As for pancreatic lipase the activity of bile salt-activated lipase is triggered by the preceding gastric lipolysis (Bernbäck et al., 1990). Among the gastrointestinal lipases, preduodenal lipase is unique in its ability to attack native milk fat globules.

Moreover gastric digestion of milk fat may have an important protective role against gastrointestinal infections. It has indeed been demonstrated that after digestion of human milk or infant formulas, an extract of the lipid fraction of the stomach showed antimicrobial activity (Isaacs et al., 1990). More specifically, medium-chain saturated and long-chain unsaturated fatty acids or mono-

acylglycerols of these fatty acids were shown to possess a high antiviral and antibacterial activity *in vitro* (Thormar et al., 1987; Isaacs et al., 1992).

1.2.1.3 Purification and characterisation of preduodenal lipases

The lingual lipase of the rat, the pregastric esterase of calf and lamb and the gastric lipase of rabbit, dog and man have been purified and biochemically characterised. All these preduodenal lipases have molecular masses between 45 and 51 kDa. Several investigators noted that the enzymes have a pronounced tendency to form aggregates. For calf pregastric esterase (Bernbäck et al., 1985) and rat lingual lipase (Field et al., 1983; Roberts et al., 1985) aggregates of 270 to 500 kDa were reported. The preduodenal lipases are glycosylated with a carbohydrate content of about 15%.

Lingual, pharyngeal and gastric lipases have common characteristics that are compatible with their activity in the stomach. All preduodenal lipases have a pH optimum between 4 and 6 and they can withstand exposure to pH 2 for some time (Moreau et al., 1988). In the upper small intestine however these enzymes are rapidly inactivated by pancreatic proteolytic enzymes (Roberts and Selma, 1988).

All preduodenal lipases are specific for triacylglycerols and none will hydrolyse the ester bonds in phospholipids or in cholesterol esters. In most cases short- and medium-chain fatty acids in the *sn*-3 position are preferentially removed. The *sn*-3 specificity results from the fact that the short and medium chain fatty acids are almost exclusively located at this position. Nevertheless long-chain fatty acids are also hydrolysed, but to a much lesser extent. Dog gastric lipase is an exception, showing a high specificity for long chain triacylglycerols (Carrière et al., 1991). Originally, it was thought that pregastric esterase could only hydrolyse short-chain fatty acids and was not active against long-chain fatty acids. This is the reason why it was called an esterase. Later however, Bernbäck (1985) demonstrated that pregastric esterase is a true lipase, with an esterase activity.

Purified gastric lipases are very sensitive to interfacial denaturation (Gargouri et al., 1986). When certain amphiphilic proteins (BSA, β-lactoglobulin) are not included in the reaction mixture before the purified enzyme is added, no activity is detected. This interfacial denaturation is irreversible since

supplementation with amphiphiles after the addition of enzyme, fails to induce activity. In contrast to this, no surface inactivation is observed with crude gastric lipase preparations. The enzyme is probably protected against denaturation by other proteins in these preparations.

1.2.1.4 Molecular structure and mechanism of action

The cDNA coding for rat lingual lipase (Docherty et al., 1985) and for the gastric lipases of man (Bodmer et al., 1987), dog and rabbit (Bénicourt et al., 1993) have been cloned. All four show a great similarity in their nucleotide and in their deduced amino acid sequences. As predicted from the cDNA sequences the unmodified enzymes have a molecular mass of about 43 kDa.

Recently the cDNA for human lysosomal acid lipase (HLAL) has also been cloned and sequenced. A close identity with preduodenal lipases was found (Anderson and Sando, 1991; Ameis et al., 1994). On the basis of this identity, and because all these enzymes are active at an acid pH, it was proposed that they belong to a distinct new lipase family: the acid lipases.

1.2.1.4.1 Preduodenal lipases are serine esterases

No sequence similarity exists among the preduodenal lipases and other mammalian or microbial lipases with the exception of a pentapeptide (Gly-Xaa-Ser-Xaa-Gly) motif which is conserved in all known lipases. The widespread distribution of this pentapeptide leads to the assumption that this amino acid sequence is essential for the action of lipases in general. As demonstrated by X-ray structure analysis and site directed mutagenesis, the serine residue in this sequence is part of the catalytic triad Ser/His/Asp.

Human and rabbit gastric lipase are readily inactivated by serine binding compounds like E600 and THL (Fig. 1-3 A) (Borgström, 1988 and Gargouri et al., 1991). E600 modified enzymes lose their activity on both water-soluble and emulsified tributyroylglycerol but are still able to bind to lipid/water interfaces (Moreau et al., 1991). These observations indicate that a serine residue participates in the catalytic mechanism of preduodenal lipases rather than in lipid binding.

Until now no information on the three dimensional structure of preduodenal lipases is available. Although crystallisation of man, rabbit and dog gastric lipase

was successful, the crystals were unsuitable for X-ray analysis (Abergel et al., 1991).

1.2.1.4.2 Preduodenal lipases are sulfhydryl enzymes

The amino acid sequences of preduodenal lipases show three conserved cysteine residues. For the gastric lipases, two of these cysteines are involved in the formation of a disulphide bridge. Blocking the free sulfhydryl group with classical sulfhydryl reagents (Fig. 1-3 B), such as DTNB or 4-PDS induces a complete loss of activity (Gargouri et al., 1988; Moreau et al., 1988; Carrière et al., 1991). More recently the inhibition of human and rabbit gastric lipase with the hydrophobic sulfhydryl reagent $C_{12:0}$-TNB was reported (Gargouri et al., 1991). Also human gastric lipase is inhibited with ajoene, a disulphide derived from garlic extracts. This is consistent with the well-known ability of garlic to lower triacylglycerol levels in the blood (Gargouri et al., 1989). All these results indicate that a free sulfhydryl group plays an essential role in the catalytic mechanism of gastric lipases. Accordingly gastric lipases are called 'sulfhydryl enzymes'.

Sulfhydryl modified lipases are still able to adsorb and penetrate lipid monolayers (Gargouri et al., 1989), even when they are unable to catalyse the hydrolysis of fats. This proves that the lipid-binding domain is functionally and topologically distinct from the catalytic site. It is proposed that modification of the sulfhydryl group only prevents the access of the substrate to the catalytic site.

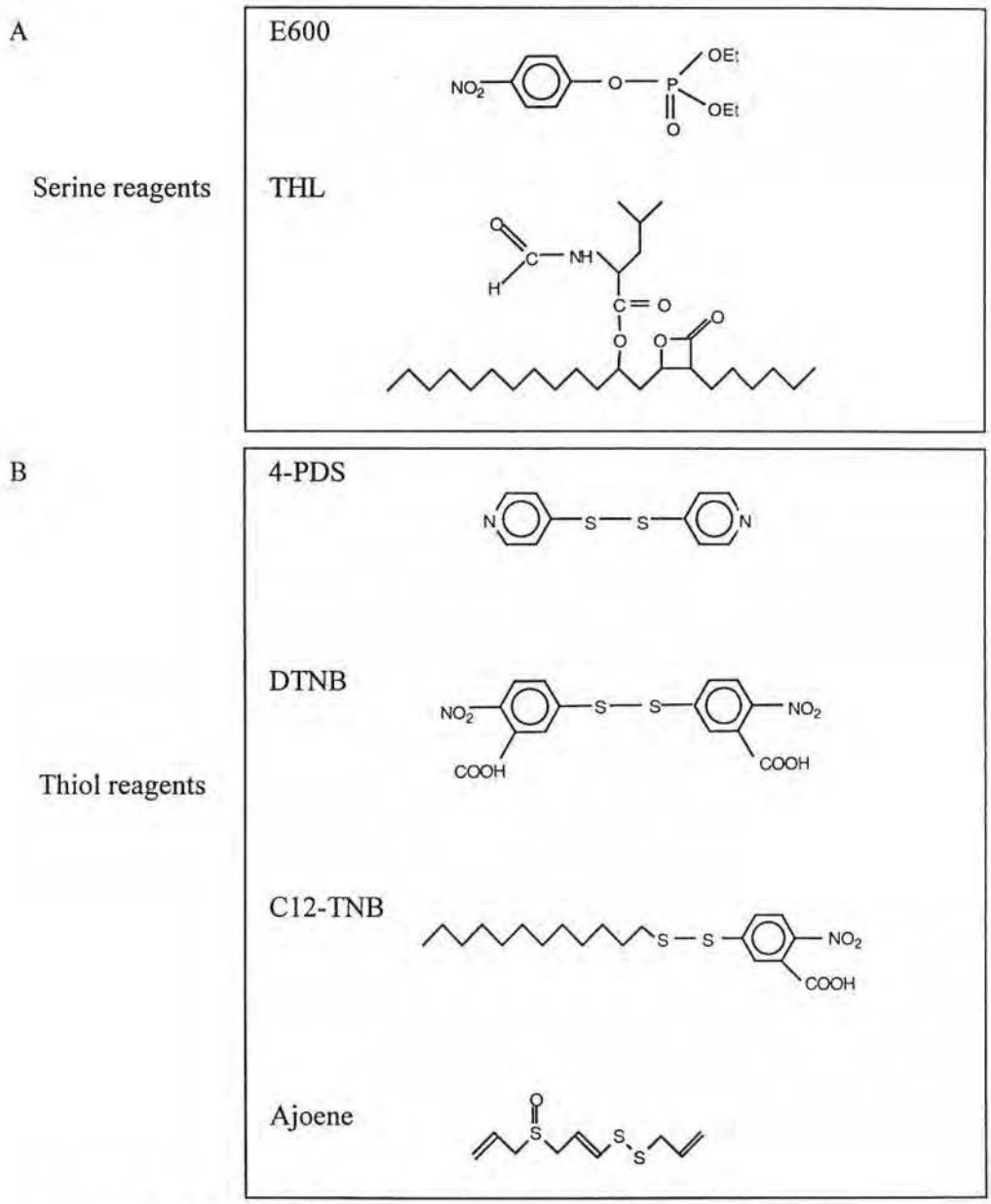

Fig. 1-2 Chemical structures of gastric lipase inhibitors
A Serine reagents; B Thiol reagents

1.2.2 Lysosomal lipase

Lysosomal lipase (Fowler and Brown, 1984) is a lipolytic enzyme present in the lysosomes. It is most active at pH 4.5-5. It is involved in the intracellular metabolism of cholesterol esters and triacylglycerols derived from LDL. The amino acid sequence of this enzyme, as deduced from the corresponding cDNA sequence, showed up to 60% identity with the preduodenal lipases. Apparently these enzymes are closely related and belong to one gene family of acid lipases (Anderson and Sando, 1991; Ameis et al., 1994).

Lysosomal lipase shows no sequence similarity with non-preduodenal lipases except for the conserved sequence around the serine residue involved in the formation of the catalytic triad. Lysosomal lipase is inhibited after chemical modification with E600 suggesting that a serine residue also may have an important role in the catalytic mechanism. In the deduced amino acid sequence six cysteines are present, three of which are conserved in the preduodenal lipases. The observation that sulfhydryl reagents inactivate the enzyme's catalytic activity implicates the involvement of at least one free cysteine residue in the catalytic mechanism of lysosomal lipase. This is consistent with the importance of one free cysteine for the catalysis by gastric lipases.

1.3 Neutral lipase family

1.3.1 Pancreatic lipase

Most of our knowledge about lipases comes from the study of pancreatic lipases. Pancreatic lipase is a digestive lipase, acting in the small intestine at neutral or alkaline pH. The enzyme uses triacylglycerols as substrates and preferentially splits at the *sn*-1 and *sn*-3 position. The digestion of dietary lipids is initiated by preduodenal lipases and then completed in the intestine by pancreatic lipase. Pancreatic lipase has been isolated and characterised from various species (Verger, 1984). It is a glycoprotein of about 50 kDa. Pancreatic lipases need colipase as a cofactor and bile salts in order to develop full activity. Bile salt

molecules accumulate at the surface of lipid droplets and colipase adheres to this bile salt layer. Colipase anchors the pancreatic lipase to the surface of the droplets.

For porcine pancreatic lipase (Sarda and Desnuelle, 1958) it was shown that the enzyme had almost no activity against methylbutyrate or triacetylglycerol but that it was activated when the concentrations of these esters exceeded the CMC and emulsions were formed (Fig. 1-4). This activation at an oil-water interface, known as interfacial activation was further generalised as a characteristic of lipases.

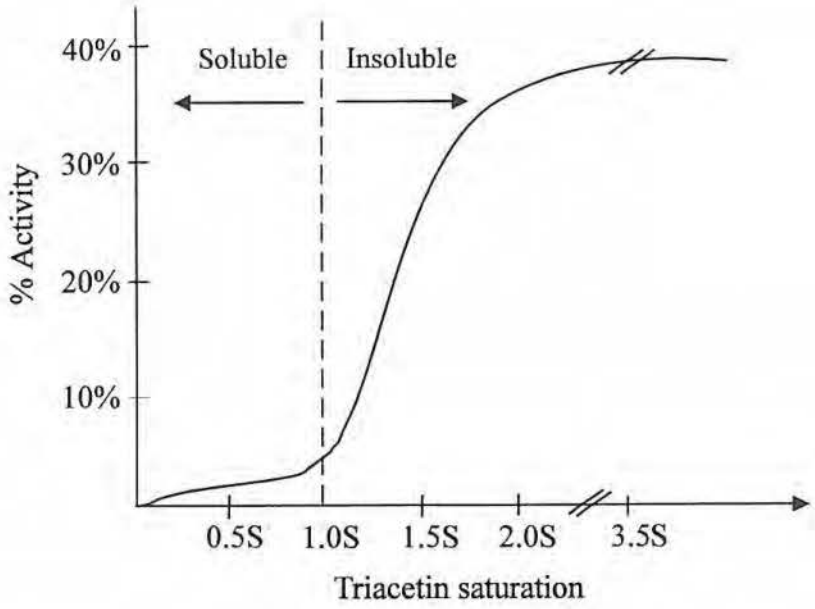

Fig. 1-3 Activity of porcine pancreatic lipase against triacetin as a function of increasing substrate concentration (Sarda and Desnuelle, 1958).

Inhibition of pancreatic lipase by E600 (Moreau et al., 1991) and THL (Ransac et al., 1991) is reported, suggesting the importance of a serine residue in the catalytic mechanism. Pancreatic lipases are also inhibited by sulfhydryl reagents (Cudrey et al., 1993, Gargouri et al., 1992) which indicates that, like the acid lipases, they need a free sulfhydryl group for their activity.

Three dimensional structures of human pancreatic lipase (Winkler et al., 1990) and of the fungal lipase of *Rhizomucor miehei* (Brady et al., 1990) reveal the presence of a Ser/His/Asp catalytic triad in both enzymes.

The importance of the three amino acids in the catalytic triad was demonstrated by site specific mutagenesis. Mutations were introduced in the cDNA of human pancreatic lipase (Lowe, 1992). After expression of the mutant cDNA's in cos7 cells it appeared that proteins mutated in one of these amino acids could still bind to an oil/water interface but had lost all activity.

Structural models of several lipases showed that the putative hydrolytic site was covered by a surface loop ("lid"), which rendered it inaccessible to substrates. The crystal structure of *Rhizomucor miehei* lipase inhibited by n-hexyl-phosphonate (Brzozowski et al., 1991) and E600 (Fig. 1-5) (Derewenda et al., 1992) demonstrated that a conformational change had occurred by which the conserved serine was exposed. It was proposed that this lipase-inhibitor complex was equivalent to the activated state of the enzyme as generated by binding to the oil/water interface. This provides a possible explanation for the interfacial activation phenomenon. The implication is that lipases act in two steps. In the first step binding of the enzyme to the oil-water interface displaces the hydrophobic loop so that the catalytic site becomes accessible. In the second step the bound substrate is hydrolysed (Derewenda et al., 1992).

This hypothesis is supported by the observation that for *Fusarium solani* cutinase, a lipase which does not exhibit interfacial activation, the catalytic site serine is not buried under a surface loop but is directly accessible (Martinez et al., 1992).

Recently the crystal structure of *Humicola lanuginosa* and *Rhizopus delemar* lipase revealed a conformational unstable lid. Different lid conformations for the same enzyme were found suggesting that the two step enzyme model of interfacial activation is an over-simplification (Derewenda et al., 1994). Clearly the mechanism of action of these enzymes at the molecular level is still not fully understood.

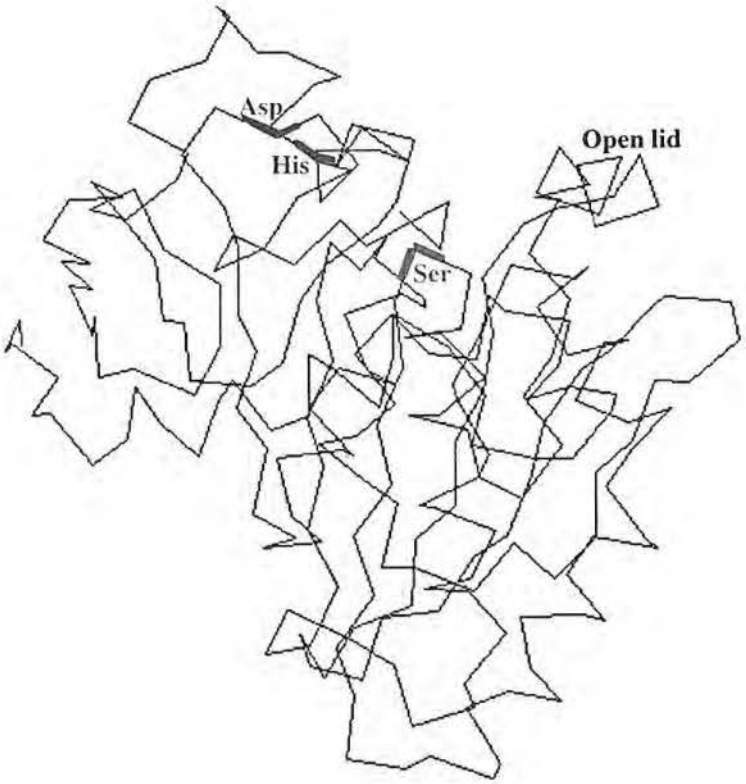

Fig. 1-4 Three-dimensional structure of Rhizomucor miehei lipase complexed with E600 (Brzozowski et al., 1991).

1.3.2 Lipoprotein lipase and hepatic lipase

The free fatty acids and monoacylglycerols produced in the intestinal lumen by the digestion of triacylglycerols are taken up into the epithelial cells of the intestinal mucosa. Within the cells the long-chain fatty acids and monoacylglycerols are used for the resynthesis of triacylglycerols. The resynthesized

triacylglycerols form small lipid droplets to which surface-active phospholipids and special proteins, called apolipoproteins, adsorb. Together with cholesterol esters they form lipoprotein complexes, termed chylomicrons. These chylomicrons are released into the intercellular space where they are taken up by lymph capillaries and finally reach the blood.

Both, lipoprotein and hepatic lipase, are involved in the metabolism of circulating lipoproteins. These enzymes are anchored at the luminal surface of vascular endothelial cells. Lipoprotein lipase is widely distributed in extrahepatic tissues including heart, skeletal muscle and adipose tissue.

Hepatic lipase (Kinnunen, 1984) and lipoprotein lipase (Wang et al., 1992) use different types of lipoproteins as substrates. Lipoprotein lipase is responsible for the hydrolysis of triacylglycerols in chylomicrons and VLDL. VLDL are transformed by the action of lipoprotein lipase into IDL. Lipoprotein lipase requires the presence of apolipoprotein CII (Apo CII) for its activity. The function of lipoprotein lipase is to direct the influx of plasma triacylglycerols in the form of fatty acids into the peripheral tissues for storage and to provide fuel for energy requirements. Hepatic lipase is mainly active on IDL, formed by lipoprotein lipase and on HDL. IDL are degraded to LDL which are subsequently taken up by endocytosis and further metabolised by lysosomal lipase. Contrary to lipoprotein lipase, hepatic lipase does not depend on any cofactor.

The cDNA sequences of some lipoprotein lipases and hepatic lipases are known. There exists a reasonable sequence identity among pancreatic lipases, lipoprotein lipases and hepatic lipases (Datta et al., 1988; Komaromy and Schotz, 1987), the overall amino acid identity between the three being about 37%. Pancreatic lipases, lipoprotein lipases and hepatic lipases are members of one gene family of neutral lipases (Raisonnier et al., 1992). Hepatic and lipoprotein lipases probably share a high degree of structural identity and possess the same catalytic mechanism as the pancreatic lipases. Site directed mutagenesis of single serine residues in human lipoprotein lipase (Emmerich et al., 1992; Faustinella et al., 1992) and in rat hepatic lipase (Davis et al., 1990) confirmed that the serine which is part of the conserved sequence is essential for the lipolytic activity of both enzymes.

1.4 Bile salt-activated lipases

In addition to pancreatic lipase, the pancreas also secretes another lipase. This enzyme is only active in the presence of bile salts and catalyses the hydrolysis of a wide range of substrates like triacylglycerols, phospholipids, vitamin esters and cholesterol esters. For this reason it is known under various names: carboxyl ester hydrolase, cholesterol esterase and carboxyl ester lipase (Wang and Hartsuck, 1993). It is assumed that this enzyme plays a secondary role in the hydrolysis of dietary triacylglycerols.

The bile salt-stimulated lipase in milk, produced by the mammary gland, has the same properties as the pancreatic enzyme (Bläckberg and Hernell, 1981). The enzyme is not inactivated in the stomach and plays an important role in milk fat digestion in the newborn.

The cDNA sequences for human bile salt-activated lipase, originating from the pancreas (Reue et al., 1991) and the mammary gland (Nilsson et al., 1990) are complete identical. No sequence identity existed with hormone sensitive lipase or with any neutral or acid lipase, except for the conserved sequence around a serine residue (Gly-Xaa-Ser-Xaa-Gly). Bile salt-activated lipase is inactivated by E600 and THL (Borgström, 1988) implying that a serine residue is essential for its lipolytic activity.

1.5 Adipose tissue lipases

Adipocytes contain a hormone sensitive lipase and a monoacylglycerol lipase (Belfrage et al., 1984). These enzymes are responsible for hydrolysing intracellular depot fats and the reaction products, glycerol and free fatty acids, are released in the blood. Hormone sensitive lipase splits the triacylglycerols preferentially at the *sn*-1 and *sn*-3 positions and the remaining 2-monoacylglycerols are hydrolysed by the monoacylglycerol lipase.

Adipose tissue lipolysis is the major regulator of the body's lipid energy supply. Hydrolysis by hormone sensitive lipase, named so because of its responsiveness to a wide range of hormones, is the rate limiting step in providing lipid fuel.

The activation of hormone sensitive lipase is regulated by a cyclic AMP mediated process. Lipolytic hormones rapidly increase enzyme phosphorylation with corresponding increase in enzyme activity, while anti-lipolytic hormones shift the ratio of the phosphorylated to the dephosphorylated form of the enzyme. Insulin is by far the most important antilipolytic hormone whereas catecholamines are the most powerful regulators of lipolysis (Coppack et al., 1994).

The cDNA for hormone sensitive lipase of the rat has been cloned (Holm et al., 1988). Except for the conserved Gly-Xaa-Ser-Xaa-Gly pentapeptide, no sequence identity with other lipases was found (Holm et al., 1994). From inhibition studies with serine binding reagents, the participation of a serine residue in the catalytic process was deduced.

1.6 Industrial and medical applications of lipases

A variety of lipases finds frequently use in the pharmaceutical, food and detergent industry for the modification of oils and fats (Bjorkling et al., 1991; Poldermans, 1987).

Lipases can perform three types of reactions: hydrolysis, synthesis and trans-(inter)esterification, the last reaction being a combination of the first two.

Furthermore lipases are to some degree specific for the type and the position of the fatty acid. *M. Miehei* lipase has a high *sn*-1,3 selectivity and is for example used to produce a cocoa butter substitute from cheaper oils, by inter-esterification. The reaction of olive oil with stearic and palmitic acids produces a mixture of triacylglycerols which is an excellent equivalent of cocoa butter. Other fungal lipases, resistant to alkaline conditions and temperatures up to 60°C are employed in detergents.

Preduodenal lipases are frequently used in flavouring. Crude calf, goat and lamb pharyngeal extracts, containing pregastric esterase, are used commercially to impart typical flavours to all sorts of cheeses and cheese-like products (Nelson 1976). Pregastric esterase produces a relatively mild piquant character. Butter-fat modified by an extract of pregastric esterase is utilised to impart dairy flavour to a wide range of processed foods like bakery and confectionery products.

Pancreatic lipase is used as a digestive aid in patients suffering from malnutrition and steatorrhea as a consequence of pancreatic insufficiency. Formerly, the missing pancreatic enzymes were replaced by the administration of porcine pancreatic extracts. This was far from satisfactory, since the enzymes therein were quickly inactivated in the stomach, with only 8% of the lipase remaining active. Over the last 10 years considerable progress has been made in the development of enteric-coated tablets of hog pancreatic extracts, resistant to an acidic environment. Even so, a marked inactivation of the administered pancreatic lipase still occurs. Supplements of gastric or lingual lipase might provide a better therapy. These supplements would indeed remain fully active in the acid environment of the stomach and would not be affected by low levels of bile salts or the absence of colipase in the intestine. One research group is currently trying to achieve the production of active dog gastric lipase on an industrial scale by way of biotechnology. This enzyme could be used to treat pancreatic insufficiency (Bénicourt et al., 1993).

1.7 Objectives

In this work the preduodenal lipase of the calf, also called pregastric esterase (PGE) is studied.

From the literature very little information on the catalytic mechanism of preduodenal lipases is available. Comparison of the biochemical properties and the primary structure of pregastric esterase, with those of other preduodenal and non-preduodenal lipases, might contribute to an understanding of the catalytic mechanism of preduodenal lipases.

In the first place a purification method for pregastric esterase was established. The enzyme was characterised and the influence of inhibitors binding to specific amino acids was studied. These data allowed determination of the functional groups essential for the lipolytic activity of pregastric esterase (Chapter 2).

Secondly, a cDNA library of calf tongue tissue from the region containing the glands of von Ebner was prepared. The cDNA coding for pregastric esterase

was amplified from this library by PCR, using primers based on conserved sequences in two other preduodenal lipases. After sequencing this cDNA, the nucleotide and deduced amino acid sequence was compared with other lipases (Chapter 3).

Due to the ability of preduodenal lipases to act in an acidic environment, they are of great interest to facilitate fat digestion in patients suffering from pancreatic insufficiency. Therefore, the production of recombinant active pregastric esterase on an industrial scale would be very helpful. Furthermore because of increasing demands for crude pregastric esterase extracts, frequently used in the food industry, the recombinant enzyme could serve as substitute.

To express functional pregastric esterase, its cDNA was cloned in expression vectors for the bacterium *E. coli* and the yeast *Pichia Pastoris*. The eukaryotic system has the advantage of carrying out post-translational modifications. To detect the recombinant enzyme, antibodies prepared against purified enzyme were used and the functionality of pregastric esterase was determined (Chapters 4 and 5).

2. Purification and characterisation of calf pregastric esterase

2.1 Introduction

In what follows we shall briefly review some of the methods that have been published for the purification of some preduodenal lipases.

Human gastric lipase (HGL) was purified from gastric juice with polyethyleneglycol 6000 precipitation, ethanol fractionation and Sephadex G-75 gel filtration by Tiruppathi and Balasubramanian (1982).

Rabbit gastric lipase (RGL) was isolated from an acetone powder of rabbit stomach by precipitation with hydrochloric acid, fractionation with ammonium sulphate, Sephadex G-100 gel filtration and cation exchange (Mono S) chromatography (Moreau et al., 1988).

Dog gastric lipase (DGL) extracted from gastric tissue was purified by cation exchange (Mono S) and anion exchange (Mono Q) chromatography, followed by gel filtration on a Superose column (Carrière et al., 1991).

Rat lingual lipase (RLL) was isolated from the serous glands of rat tongue by ammonium sulphate precipitation followed by acetone precipitation and hydrophobic interaction chromatography on ethyl agarose (Field and Scow, 1983).

Lamb pregastric lipase (LPGL) was purified from pharyngeal tissue. An aqueous extract was subjected to anion exchange chromatography on DEAE-cellulose, followed by chromatography on Hydroxyl Apatite-Ultrogel and gel filtration on Ultrogel AcA-54 (De Caro, personal communication).

For the purification of calf pregastric esterase (PGE) two methods have been published so far. Sweet et al. (1985) purified pregastric esterase from delipidated gullet tissue by acid precipitation, DEAE-Sephacel anion exchange chromatography and repeated gel filtration on Sephadex G-100. In another study (Bernbäck et al., 1985) pregastric esterase was purified from calf pharyngeal tissue

by Octyl Sepharose, lentil lectin affinity chromatography and gel filtration on ACA-44.

None of the last two methods gave satisfactory results in our hands. For this reason we have worked out an original method for the purification of calf PGE.

2.2 Experimental procedures

2.2.1 Determination of PGE activity

2.2.1.1 pH-stat method

For quantitative purposes lipase activity was measured titrimetrically at room temperature in a pH-stat (Metrohm) at pH 5.5, using a 2.5 ml tributyrin emulsion as the substrate. This emulsion was prepared as follows: 0.125 ml tributyrin was added to 2.5 ml of 5% gum arabic, 0.9% NaCl and 2 μM BSA and mixed for 20 sec at maximum speed with a Sorvall mixer. The pH in the reaction mixture was kept constant by the continuous addition of 0.005 N NaOH from an automatic burette. The amount of NaOH added was recorded for at least 15 min. This amount is directly proportional to the amount of released butyric acid. One unit of activity (U) is defined as the amount of enzyme that liberates 1 μmole of butyric acid per min.

Specific activity is expressed as U per mg protein. Protein concentrations were determined by the BCA method (Pierce).

2.2.1.2 Colorimetric method

A stock solution of 50 mM p-nitrophenyl butyrate (PNPB) was prepared by dissolving 10.45 mg PNPB in 1 ml acetonitrile. Just before use, the PNPB stock was diluted 1/55 (v/v) in PBS. 10 μl of the sample was incubated with 180 μl of freshly diluted substrate solution in microtiter plate wells. The hydrolysis of PNPB was monitored for 10 min by measuring the colour development at 405 nm. This method was used as a fast qualitative test to determine the enzyme activity in

column fractions. It has to be noted that in this test we actually measure the esterase activity of PGE.

2.2.2 Purification of PGE

2.2.2.1 Tissue preparation

Pharyngeal tissue of 24 weeks old calves was obtained from a local slaughterhouse. The tissue was cut in 1 cm pieces after removing most of the muscle. PGE extracts were prepared by soaking 40 g of pharyngeal tissue in 100 ml phosphate buffer (28 mM KH_2PO_4, 39 mM Na_2HPO_4, 15 mM NaCl and 1 mM Benzamidine, pH 7) at 4°C for one hour. This buffer will be referred to as "extraction buffer". After centrifugation for 15 min at 12000 *g* the tissue pellet was extracted three more times with the same buffer. The supernatants of the successive extractions were pooled and used for subsequent purification. All column chromatographic steps were performed with an HPLC apparatus from Pharmacia.

2.2.2.2 Anion exchange chromatography

Anion exchange chromatography was carried out on a 50 ml $Q_{FAST\ FLOW}$ Sepharose column (26 mm x 20 cm, Pharmacia) equilibrated with extraction buffer. The tissue extract was loaded at a flow rate of 4 ml/ min. The column was washed with two column volumes of extraction buffer and the bound material was eluted with a linear NaCl gradient (0 to 0.5 M in extraction buffer). Fractions of 12 ml were collected, lipolytic activity was measured by colorimetry with the PNPB method (2.2.1.2) and the active fractions were pooled.

2.2.2.3 Ammonium sulphate precipitation

The pooled $Q_{FAST\ FLOW}$ fractions were concentrated by precipitation at 50% ammonium sulphate saturation. An equal volume of saturated ammonium sulphate solution was slowly added at 4 °C with continuous stirring. After incubation for 30 min at 4 °C the mixture was centrifuged 15 min at 12000 *g*. The precipitate was

resuspended in 10 ml PBS and centrifuged again. The clear supernatant was used for further purification by gel filtration.

2.2.2.4 Gel filtration chromatography

The supernatant from the previous step (about 10 ml) was loaded on a Sephacryl S-200 HR column (26 mm x 85 cm, Pharmacia) equilibrated with PBS. Elution was carried out with PBS at a flow rate of 2 ml/min and fractions of 8 ml were collected. Lipolytic activity was measured with PNPB as the substrate and the active fractions were pooled. The column was calibrated with BSA (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa) as molecular weight markers.

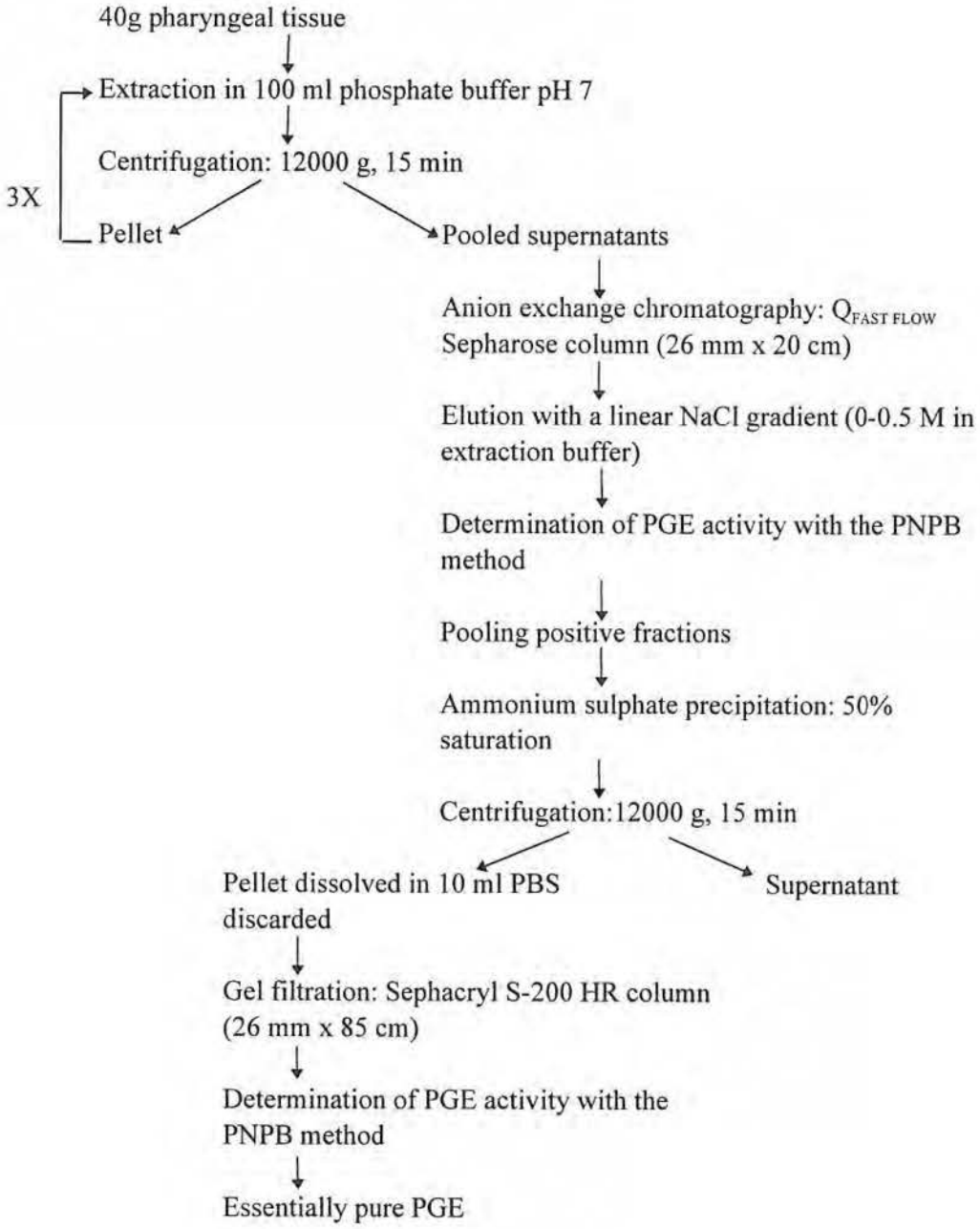

Fig. 2-1 PGE purification flow scheme

2.2.3 SDS-PAGE

Discontinuous SDS-polyacrylamide electrophoresis was performed according to the standard procedure of Laemmli (1970). A 5% stacking gel (5% acrylamide, 0.16% bisacrylamide, 125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.034% APS and 0.1% TEMED) and a 10% or 12.5% separating gel (10% or 12.5% acrylamide, 0.27% N,N'-methylenebisacrylamide, 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.05% APS en 0.033% TEMED) were used. Before application, the samples were boiled for 5 min in the presence of sample buffer (10% glycerol, 2% β-mercaptoethanol, 2% SDS, 0.0025% bromophenol blue and 62 mM Tris-HCl pH 6.8). 25 mM Tris-192 mM glycine buffer with 1% SDS, pH 8.3 was the running buffer, and the gels were run in a LKB Midget electrophoresis system.

Protein bands on the gels were revealed with Coomassie brilliant blue (0.1% Coomassie, 10% glacial acetic acid, 25% methanol) or by silver staining.

Phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa) were used as molecular weight standards.

2.2.4 Antibody production

For the production of polyclonal antibodies (pAbs) against PGE, one rabbit was given a first injection of 160 µg of purified enzyme together with Freund's complete adjuvant. Monthly booster injections were given with the same amount of purified PGE alternately in incomplete adjuvant or PBS. After the second booster injection the animals were bled every 2 weeks and the serum was recovered.

Monoclonal antibodies (mAbs) against PGE were prepared according to the procedure of Köhler and Milstein (1975) (Fig. 2-2). A female BALB/c mouse (8-10 weeks old) was immunised two times with PGE. First 12 µg purified PGE emulsified in Freund's complete adjuvant was injected intraperitoneally. After one month a second immunisation with 25 µg PGE in PBS was performed. Four days

later the spleen cells were isolated and fused with myeloma SP2/0 cells at a ratio of 2:1 with 50% PEG 4000. The hybridomas were grown for one week on selective HAT (hypoxanthin/aminopterin/thymidin) medium in microtiter tissue culture plates (Nunclon, Nunc) containing a feeder layer of macrophages. The culture supernatant of each well was tested for anti-PGE activity by ELISA. Positive hybridomas were subcloned using the limiting dilution technique (0.5 cell per well) and screened again. Subcloning was repeated until the clones were pure. To make greater quantities of the mAbs ascites was produced. For ascites production 1-2 x 10^6 hybridoma cells were injected intraperitioneally in mice and ascitic fluid was collected.

For the ELISA, microtiter plates (Maxisorb, Nunc) were coated overnight at 4 °C with 1 µg purified PGE per well in coating buffer (15 mM Na_2CO_3, 26 mM $NaHCO_3$, 3 mM NaN_3, pH 9.5). The plates were washed three times (washing buffer: 0.025% Tween 80, 0.15 M NaCl, 0.025 M Tris pH 7.2) and incubated with 100 µl/well of the hybridoma supernatants for 3 hours at 37 °C. After three washings, mAbs against PGE were detected by incubation with 100 µl per well rabbit anti-mouse-HRP antibodies (1/1000; Dako P161) for 1 hour at 37 °C. Finally, the plates were washed three times with washing buffer and incubated with 100 µl per well OPD solution (0.12% OPD, 0.1% H_2O_2 in 48 mM citric acid, 103 mM NaH_2PO_4 pH 5). Colour development was stopped by the addition of 100 µl 2 N H_2SO_4 per well and the absorbance was measured at 492 nm (MPR_{A4} ELISA reader, Eurogenetics).

Rabbit pAbs and mAbs against HGL were a gift from the laboratory of Prof. R. Verger, CNRS Marseilles.

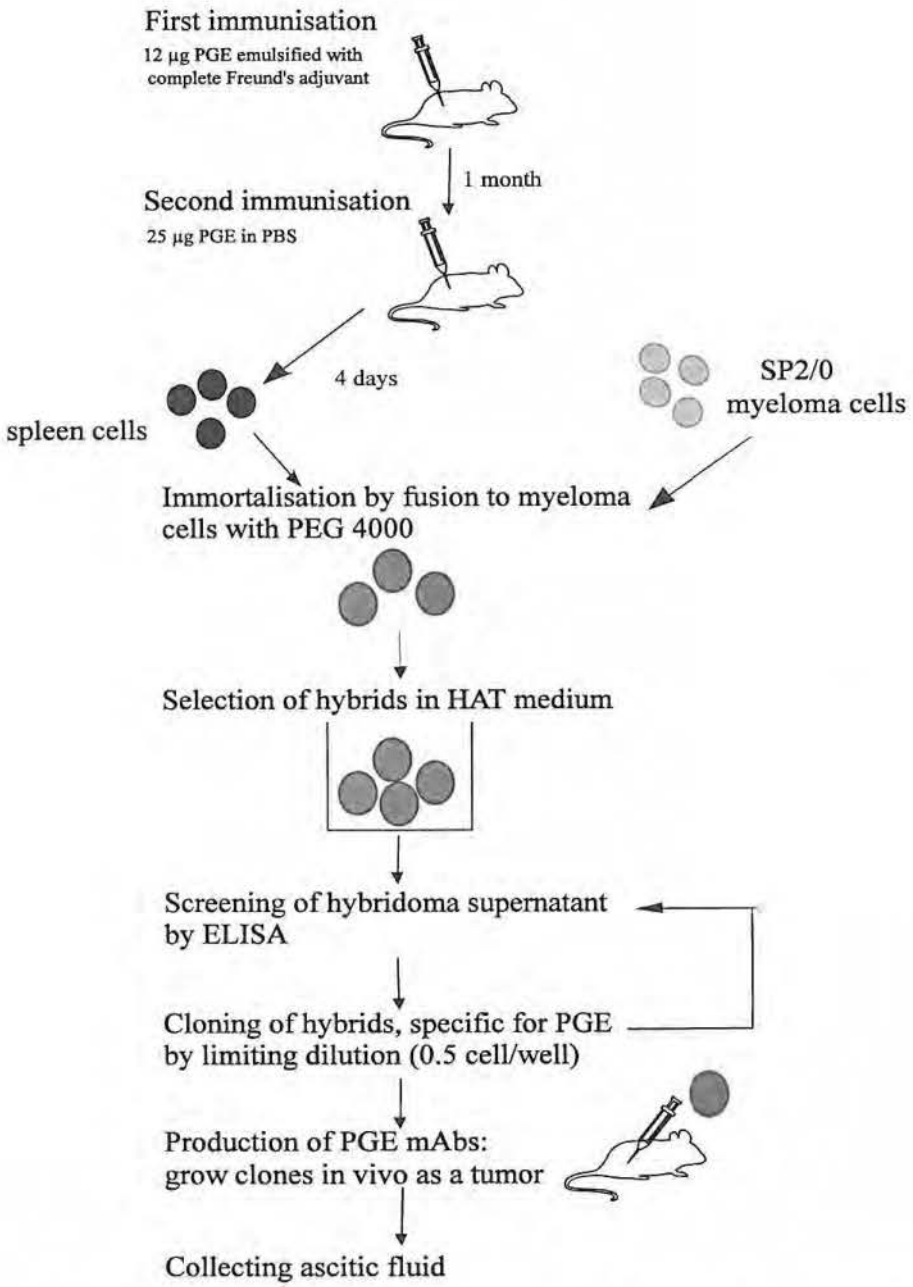

Fig. 2-2 Scheme for the production of monoclonal antibodies against PGE.

2.2.5 Immunological techniques

2.2.5.1 Western blotting

After SDS-PAGE the separated proteins were electrophoretically transferred to a PVDF membrane using a semi-dry blotting apparatus (W.E.P. company). Blotting was carried out for 45 min at a constant current of 2 mA/cm^2 , using 25 mM Tris, 192 mM glycine, 20% methanol (pH 8.3) as a buffer.

After transfer, the proteins were visualised with Ponceau-S. The lane with the molecular weight markers was cut out and stained with Coomassie brilliant blue. To block the remaining unoccupied sites the membrane was kept for at least one hour in PBS, 0.2% Tween 20 and 10% non-fat dry milk. After the blocking step the membrane was incubated overnight with the antiserum. The antiserum was diluted in PBS, 0.2% Tween 20 and 3% non-fat dry milk. The dilution factor varied with the antiserum. The membrane was rinsed three times with 0.02% Tween 80 in PBS and incubated for one hour with a second antibody conjugated with horse radish peroxidase (rabbit anti-mouse-HRP, Dako P161; swine anti-rabbit-HRP, Dako P217) or alkaline phosphatase (goat anti-mouse-AP, Dako D486; swine anti-rabbit-AP, Dako D306). The conjugated antibodies, were diluted 1/1000 in the same buffer as the antiserum. After three washings with PBS/Tween 80 the blots were incubated with the substrate. Two substrates were used: DAB (0.05% DAB, 0.03% H_2O_2 in PBS) with peroxidase and BCIP/NBT (0.016% BCIP and 0.03% NBT in 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$ pH 9.5) with alkaline phosphatase.

2.2.5.2 Immunoprecipitation

The immunoglobulin fraction from the polyclonal antiserum against PGE and from the preimmune serum, was purified by precipitation with 33% ammonium sulphate. The precipitated antibodies were resuspended in PBS, in their original volume and dialysed overnight against PBS.

The antiserum was coupled to protein A-Sepharose in a 1/1 (v/v) ratio. The mixture was incubated for 1 hour at room temperature. Different amounts of anti-

PGE-protein A-Sepharose beads (0µl, 5µl, 10µl, 15µl, 30µl and 45µl) were incubated with 50 µl purified PGE (50 µg/ml). After overnight incubation at 4 °C residual enzyme activity in 10 µl of the supernatant was determined with the colorimetric method (PNPB).

2.2.5.3 Immunocytolocalisation

Localisation of PGE in the calf's tongue and pharynx with antibodies was performed on frozen sections (5 µm). The slides were blocked for one hour at room temperature with 0.1% BSA in PBS and incubated overnight at 37 °C with hybridoma supernatant, diluted 1/2 in PBS. After washing three times with PBS + 0.5 M NaCl, the slides were incubated for two hours at 37 °C with a second antibody conjugated with horse radish peroxidase (rabbit anti-mouse-HRP) diluted 1/250 in PBS. For staining, a stock solution of 0.4% 3-amino-9-ethyl-carbazole in N,N-dimethylformamide was prepared. Before usage the stock solution was diluted 1/15 with 0.1 M sodium acetate buffer pH 5.2 and 15 µl H_2O_2 (30%) was added. Finally the slides were counterstained for 1 min with Mayer's haematoxylin (Sigma).

2.2.6 Deglycosylation of PGE

N-linked carbohydrates were removed by overnight incubation of 50 µl purified PGE (50 µg/ml) with 0.2 units endoglycosidase F (Boehringer) at 37°C. The samples were then analysed by Western blotting.

2.2.7 Influence of DTNB, 4-PDS, $C_{12:0}$-TNB, $C_{12:0}$ and Hg^{2+} ions on PGE activity

1 ml (200 µg) of purified PGE was preincubated with DTNB, 4-PDS, C12:0-TNB or C12:0 at final concentrations of 0.45 mM, 0.4 mM, 0.4 mM and 0.5 mM, respectively (at least a 100 molar excess). After different incubation times 50 µl samples were taken for determination of the residual lipase activity with tributyrin in the pH-stat. The influence of Hg^{2+} ions was determined by

incubating purified PGE with 0.33 mM or 33 mM $HgCl_2$. After 30 min the remaining enzyme activity was measured.

2.2.8 Influence of E600 and THL on PGE activity

E600 was dissolved at a concentration of 40 mM in acetonitrile. From this solution a final concentration of 0.48 mM was added to a 1 ml aliquot of PGE (200 µg). This corresponds to molar ratio of 120/1. The residual lipase activity in 50 µl samples was measured at different times in the pH-stat with tributyrin as substrate.

For THL a stock solution of 20 mM in ethanol was prepared. THL was added in a 100 molar excess to partially purified PGE ($Q_{FAST\ FLOW}$) and residual lipase activity was measured in the pH-stat.

2.2.9 Influence of β-mercaptoethanol on PGE activity

The influence of a final concentration of 56 mM β-mercaptoethanol on lipolytic activity was determined. An aliquot of partially purified PGE ($Q_{FAST\ FLOW}$) was incubated with β-mercaptoethanol and residual activity was measured in 50 µl samples at different times, using the pH-stat method.

2.2.10 Tryptic cleavage and NH_2-terminal sequence analysis of PGE

1 ml of purified PGE (200 µg) was cleaved with 35 U of TPCK-treated trypsin attached to beaded agarose (Sigma T4019) for 25 min at room temperature. Samples of tryptic digested PGE were subjected to Western blotting (section 2.2.5.1) under reducing and non-reducing (without β-mercaptoethanol) conditions.

To determine the NH_2-terminal sequences, the tryptic fragments were blotted on a Problott membrane (Applied Biosystems) and coloured by Coomassie blue. Transfer was carried out under the same conditions as described in section 2.2.5.1 except that a CAPS buffer (10 mM CAPS with 10% methanol pH 11) instead of a Tris-glycine buffer was used as transfer buffer. Automated Edman protein degradation was performed by Prof. L. Moens (UIA, Antwerp).

2.3 Results and discussion

2.3.1 Purification and characterisation of pregastric esterase

PGE secretion mainly occurs on the dorsal part of the tongue (von Ebner's glands) and in the pharynx (Moreau et al., 1988). Initial attempts to purify PGE, by column chromatography of extracts from delipidated tongue tissue, were not successful. These extracts were very viscous, low in enzyme activity and difficult to handle.

Pharyngeal tissue proved much more suitable as starting material for the purification of PGE. Four different buffers were tested for extraction of the tissue:

(1) 0.1 M glycine with 0.1 M NaCl pH 3.5
(2) 20 mM sodium acetate pH 5
(3) 28 mM KH_2PO_4, 39 mM Na_2HPO_4 and 15 mM NaCl pH 7
(4) 10 mM Tris with 3.3 mM $CaCl_2$ pH 8.5.

Since maximal enzyme activity was preserved with the phosphate buffer, this buffer was adopted for PGE extraction.

The elution pattern obtained by anion exchange chromatography of the pooled extracts of pharyngeal tissue on a $Q_{FAST\ FLOW}$ column is shown in fig. 2-3. The fractions eluting between 175 mM and 250 mM NaCl (fractions 19-34) were pooled. Their combined activity accounted for 30% of the PGE activity initially loaded on the column.

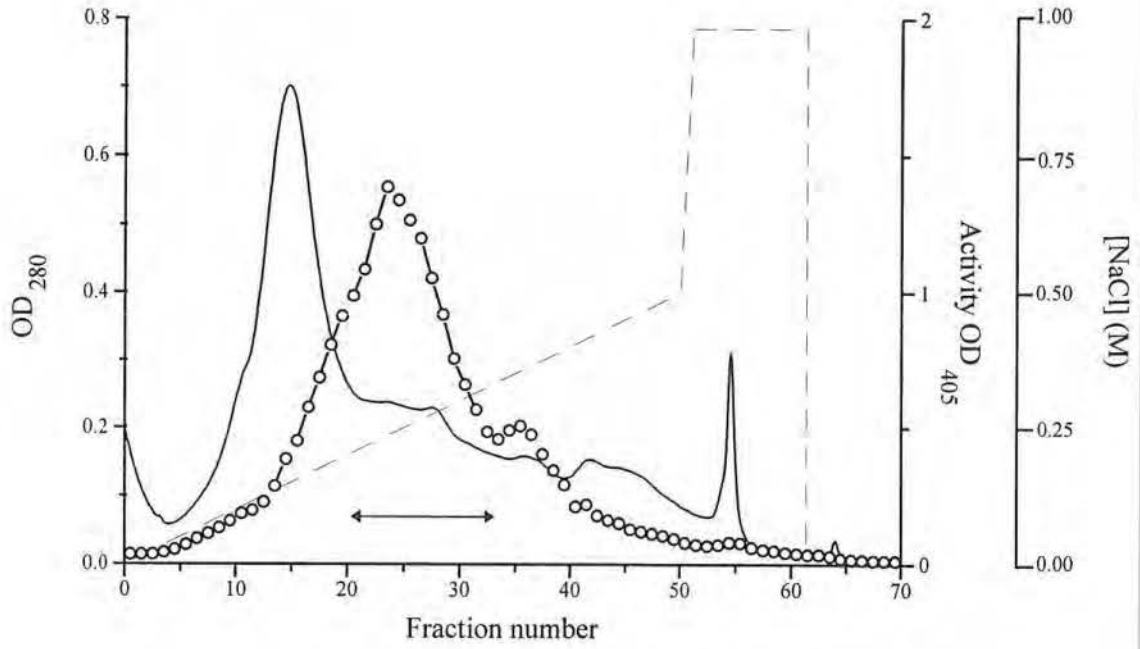

Fig. 2-3 Ion exchange chromatography of a pharyngeal PGE extract on a $Q_{FAST\ FLOW}$ column (26 mm x 20 cm).
Elution was carried out with a linear NaCl gradient of 0 to 0.5 M (– –).
Protein (——) was determined by monitoring the OD_{280} of the effluent.
PGE activity (-○-) was measured with PNPB. Pooled fractions (19-34) are indicated by an arrow(⟷).

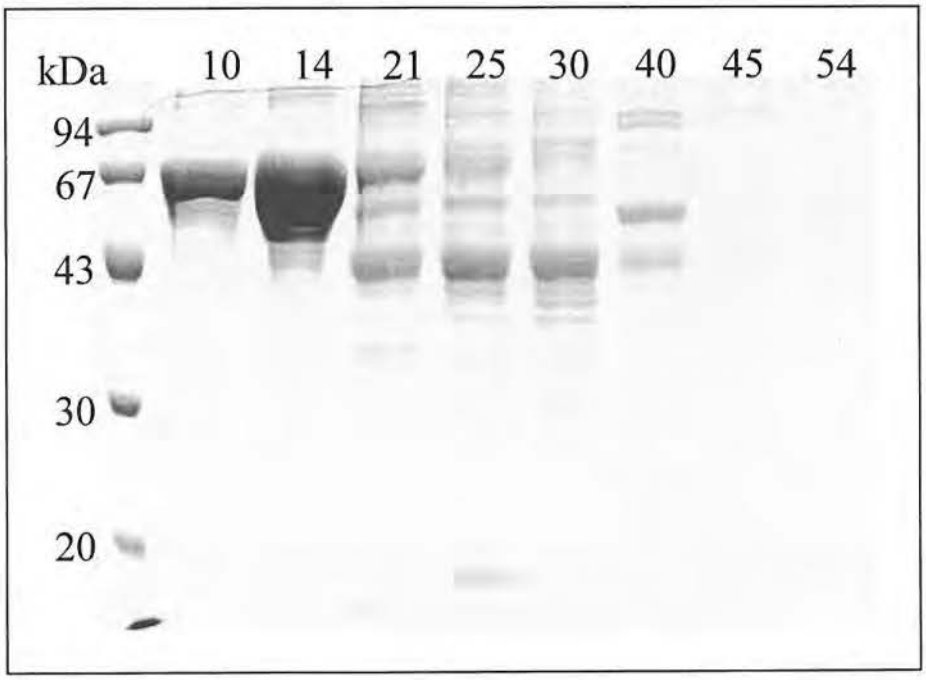

Fig. 2-4 SDS-PAGE analysis of $Q_{FAST\ FLOW}$ fractions

Electrophoresis was performed on a 10% gel and protein bands were stained with Coomassie blue. Lane 1: molecular weight markers. Lane 2-9: 30 µl of $Q_{FAST\ FLOW}$ fractions. The fraction number is indicated above each lane.

As can be seen from SDS gel in fig. 2-4, the pooled fractions contain next to PGE several other proteins. In fractions 19-21 the main contaminant is BSA. At 50% ammonium sulphate saturation almost all PGE activity in the pooled fractions was precipitated, while BSA remained largely in solution. The precipitate was resuspended in 10 ml PBS. The supernatant obtained after centrifugation of this suspension contained 26% of the lipase activity in the original extract.

Chromatography of this supernatant on a Sephacryl S-200 gel filtration column gave the elution profile shown in fig. 2-5. Fractions 27-31 contained pure PGE as judged by SDS-PAGE followed by silver staining (Fig. 2-6). These

fractions were combined and used for further experiments (production of pAbs and mAb' and inhibition studies).

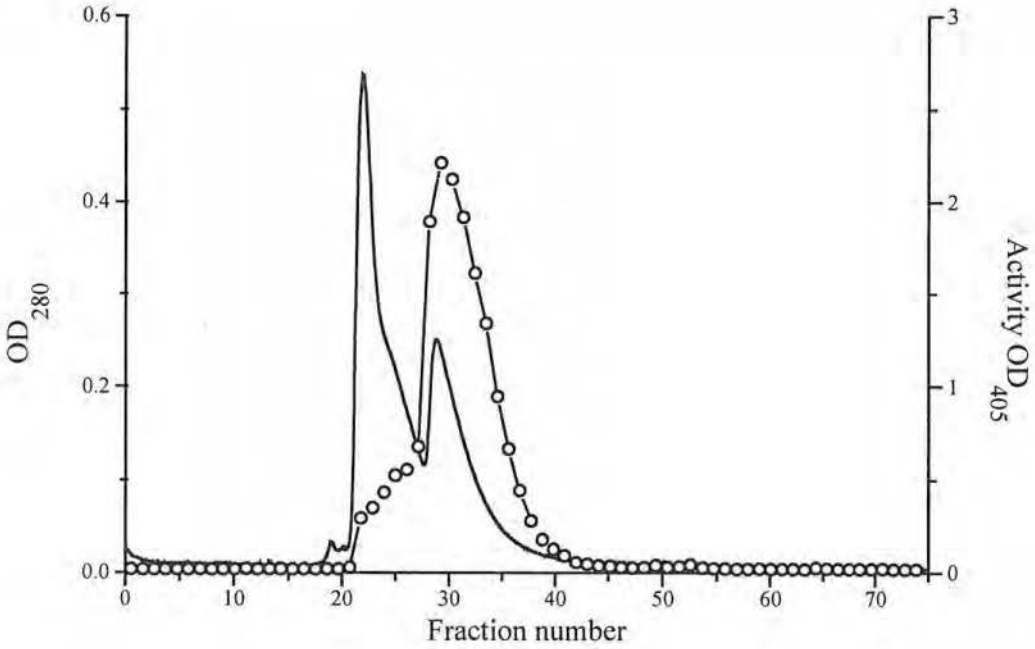

Fig. 2-5 Elution profile of PGE activity from a Sephacryl S 200 column (26 mm x 85 cm).

Pooled $Q_{FAST\ FLOW}$ fractions were precipitated with 50% ammonium sulphate and resuspended in 10 ml PBS before they were loaded on the column. Measurement of the OD_{280} was used for monitoring the protein (——) content in the effluent. PGE activity (-O-) in the fractions was determined with PNPB.

Table 2-1 shows the complete purification flowsheet. A 13-fold purification was achieved with an overall recovery of 14% of the enzyme activity present in the original tissue extract. About 6.3 mg purified PGE were obtained starting from 40 g fresh calf pharyngeal tissue.

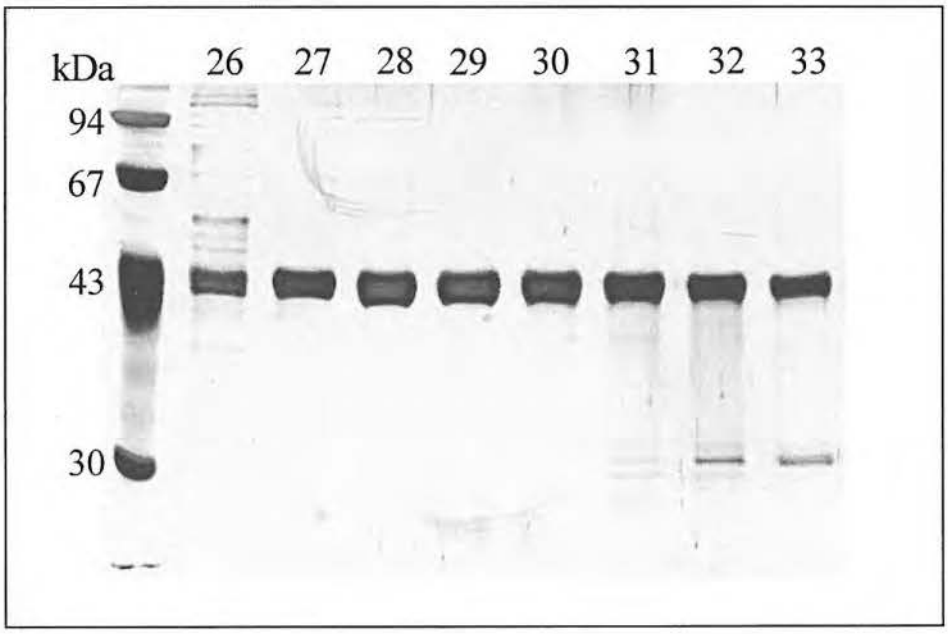

Fig. 2-6 SDS-PAGE analysis of S200 Sephacryl fractions

Electrophoresis was performed on a 10% gel and protein bands were revealed by silver staining. Lane 1: low molecular weight markers. Lane 2-9: 15 µl of S200 Sephacryl fractions. The fraction number is mentioned on top of each lane.

Table 2-1 Flowsheet of the purification of calf pregastric esterase

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg-^{1})	Activity recovered (%)	Purification factor
Crude extract	11933	604	20	100	1
$Q_{FAST\ FLOW}$ chromatography	3603	66	55	30	2.75
$(NH_4)_2SO_4$ precipitation	3114	28	111	26	5.55
Sephacryl S-200 gel filtration	1639	6.3	260	14	13

Purified PGE appeared as a single band on SDS-PAGE with a molecular mass of 50 kDa (Timmermans et al., 1996). The molecular mass determined by gel filtration was about 46 kDa. These results correspond to earlier findings. Sweet et al., (1984) determined a molecular mass for PGE of 49 kDa on SDS-PAGE and 42 kDa on gel filtration. Bernbäck et al., (1985) found a molecular mass of 55 kDa on SDS-PAGE and 46 kDa on gel filtration. All previously published purification procedures for PGE and other pregastric lipases include a lipid extraction step to release the enzyme from lipoprotein complexes. Bernbäck et al., (1985) described the presence of inactive PGE aggregates, while Field and Scow (1984) reported the existence for RLL of lipoprotein aggregates with a molecular mass of 500 kDa. In our purification procedure for PGE no enzyme aggregation was encountered, although no lipid extraction was performed.

2.3.2 Immunological characterisation

2.3.2.1 PGE polyclonal and monoclonal antibodies

The serum from the rabbit, immunised with purified PGE (pAbs) gave a distinct reaction with PGE by ELISA and Western blot (Fig. 2-7).

Three pure mAbs (3C9C7, 3A8A9 and 3F7H3) were selected and cloned using the limiting dilution technique. These clones were cultured for storage and for the production of ascites.

Isotyping the mAbs showed that 3C9C7 and 3F7H3 belong to the IgG1 type whereas 3A8A9 is an IgM antibody.

The mAbs 3C9C7 (Fig. 2-7) and 3F7H3 gave a distinct reaction with the enzyme as revealed both by ELISA and Western blot analysis while 3A8A9 showed only a reaction by ELISA.

Table 2-1 Reaction of PGE antibodies with PGE in Western blot and ELISA.

PGE antibodies	ELISA	Western blot
pAbs	+	+
3C9C7	+	+
3A8A9	+	-
3F7H3	+	+

2.3.2.2 HGL monoclonal and polyclonal antibodies

Anti-HGL polyclonal and monoclonal antibodies (4-3, 25-4, 35-2, 218-13) were obtained from Prof. R. Verger. All these antibodies recognised HGL in ELISA as well as in Western blot.

mAbs 4-3, 25-4 and 35-2 possess the capacity to inhibit HGL while 218-13 has no inhibitory effect (Aoubala et al., 1993). The three inhibitory mAbs reduce the lipid binding capacity of HGL (Ivanova et al., 1993).

Table 2-2 Reaction of HGL antibodies with HGL in Western blot and ELISA

HGL antibodies	ELISA	Western blot	Inhibition
pAbs	+	+	+
4-3	+	+	+
25-4	+	+	+
35-2	+	+	+
218-13	+	+	-

2.3.2.3 Cross-reactivity of HGL and PGE antibodies

Fig. 2-7 shows a clear cross-reaction of the anti-PGE pAbs and the mAb 3C9C7 with RGL and HGL.

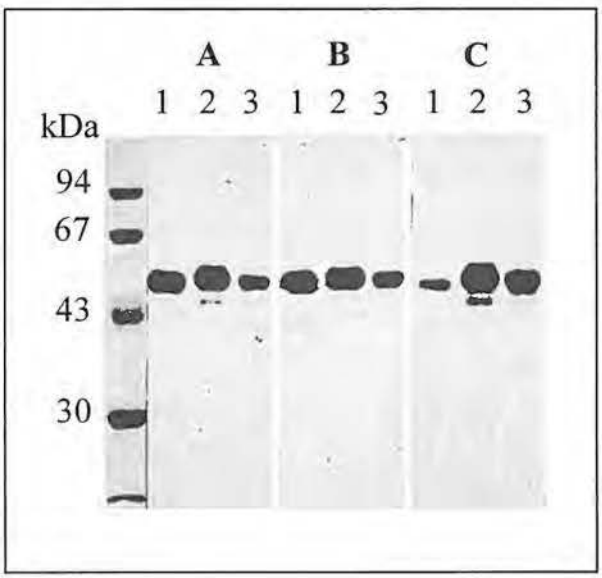

Fig. 2-7 Western blot analysis of PGE, HGL and RGL with mAb 3C9C7, rabbit anti-PGE and rabbit anti-HGL antibodies.
5 µg of PGE, HGL and RGL were loaded respectively in lane 1, 2 and 3. Proteins were detected with (A) pAb's against PGE (1:1000); (B) mAb 3C9C7 (1:2000) and (C) pAb's against HGL (1:1000). The second antibodies (swine anti-rabbit-HRP and rabbit anti-mouse-HRP) were diluted 1:1000. The Western blot was developed using DAB as the substrate.

Anti-HGL pAbs and mAb 218-13 gave a distinct reaction with PGE in Western blot (Fig. 2-7) and ELISA (Fig. 2-8). mAb 35-2 gave only a positive reaction in ELISA while mAbs 4-3 and 25-4 showed only a clear cross-reaction with PGE in Western blot.

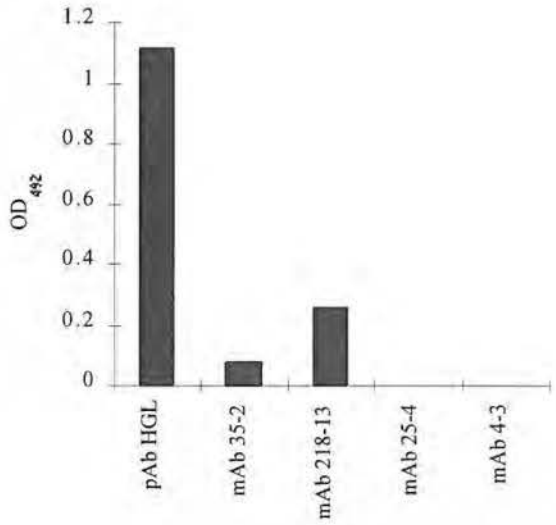

Fig. 2-8 Reaction of anti-HGL antibodies with PGE, as tested by ELISA.

These results suggest the presence of several homologous epitopes within the preduodenal lipases which is to be expected because of the considerablegreat sequence identity among these enzymes.

2.3.2.4 Immunoprecipitation with anti-PGE polyclonal antibodies

Rabbit anti-PGE pAbs were immobilised on protein A-Sepharose beads and were tested for their capacity to precipitate PGE activity. The residual PGE activity in the supernatant was measured. Fig. 2-9 clearly shows that PGE formed complexes with the immobilised antibodies. The activity in the supernatant sharply declines with increasing amounts of immobilised anti-PGE pAbs. More than 90% of the PGE activity is precipitated by the addition of 15 µl anti-PGE coated protein A-Sepharose beads. Immunoprecipitation with protein A-Sepharose beads coated with rabbit preimmune serum showed only a minor (10%) precipitation of PGE activity .

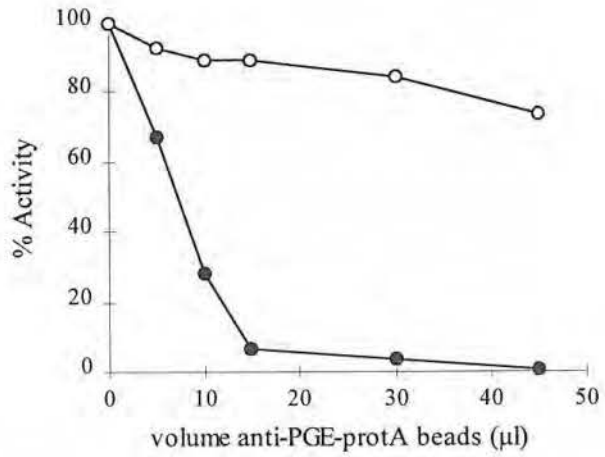

Fig. 2-9 Immunoprecipitation of PGE with anti-PGE polyclonal antibodies
Immunoprecipitation with protein A-Sepharose beads coated with rabbit preimmune serum (-○-).
Immunoprecipitation with rabbit anti-PGE serum coupled to protein A-Sepharose (-●-).

2.3.2.5 Immunocytolocalisation

Figure 2-10 shows a photograph of a 0.5µm thin frozen section of the region containing the circumvallate papillae of the calf tongue, after incubation with mAb 3C9C7. As can be seen in the enlarged part of the section distinct positive reaction of this mAb is given by the serous glands located just below the circumvallate papillae. Obviously these glands are secreting PGE.

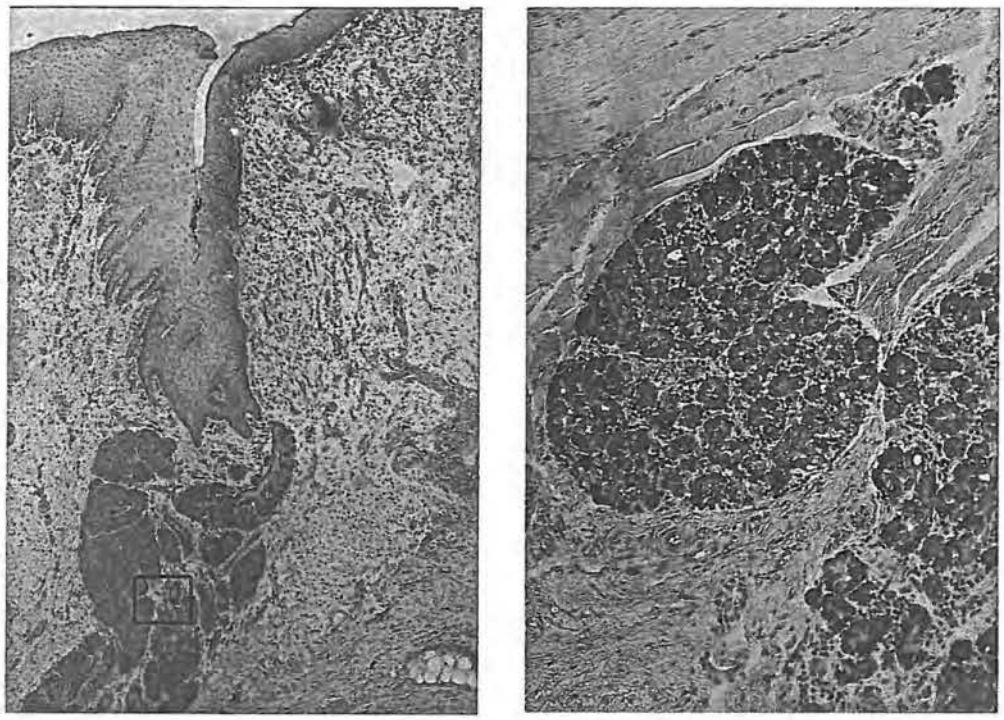

Fig. 2-10 0.5 µm frozen section through the circumvallate papillae of the calf tongue, incubated with mAb 3C9C7

2.3.3 Deglycosylation of PGE

The effect of removing N-linked carbohydrates in PGE by endoglycosidase F treatment is shown in fig. 2-11. Native PGE migrates as a protein of 50 kDa on SDS-PAGE. Endoglycosidase F treated PGE shows two bands. The major band probably represents the completely deglycosylated form of PGE and corresponds to a protein with a molecular mass of about 40 kDa. This demonstrates that PGE is a glycoprotein with an N-linked carbohydrate content of 20% of the total molecular mass of the native protein.

After endoglycosidase F treatment PGE retained only 20% of its original activity. This shows the importance of N-glycosylation for the lipolytic activity of the enzyme. The remaining activity could be explained by incomplete deglycosylation. The fact that N-glycosylation is essential for the lipolytic activity of lipases is supported by other reports. Changing the potential N-glycosylation sites of human lipoprotein lipase and human hepatic lipase by site directed mutagenesis resulted in both cases in the abolishment of the enzyme activity (Ben-Zeev et al., 1994). For HGL a decrease of 50% in activity was observed after deglycosylation (Moreau et al., 1992).

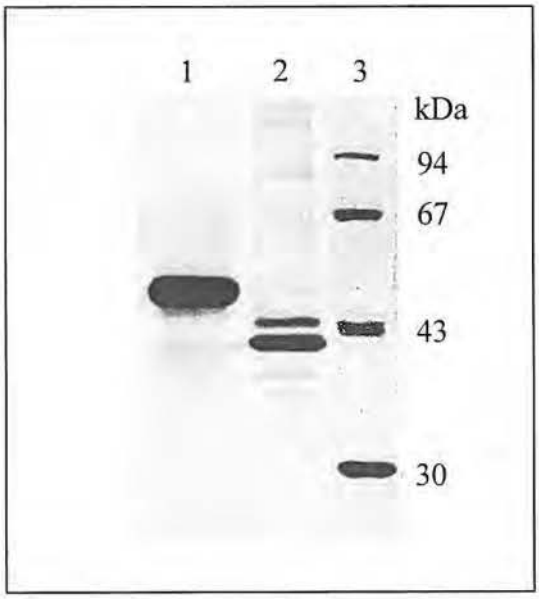

Fig. 2-11 Western blot analysis of PGE treated with endoglycosidase F
lane 1: native PGE
lane 2: deglycosylated PGE
lane 3: molecular weight standards
PGE was detected with pAb's against PGE (1:1000). Visualisation was performed with swine anti-rabbit-HRP (1:1000) and DAB as a substrate.

2.3.4 Optimalization of assay conditions for PGE activity

2.3.4.1 pH optimum

Fig. 2-12 shows the pH activity profile for purified PGE acting on a tributyrin emulsion. The optimal pH is 5.5. This is in a good agreement with the value of pH 4 to 6 found so far for all preduodenal lipases (Moreau et al., 1988).

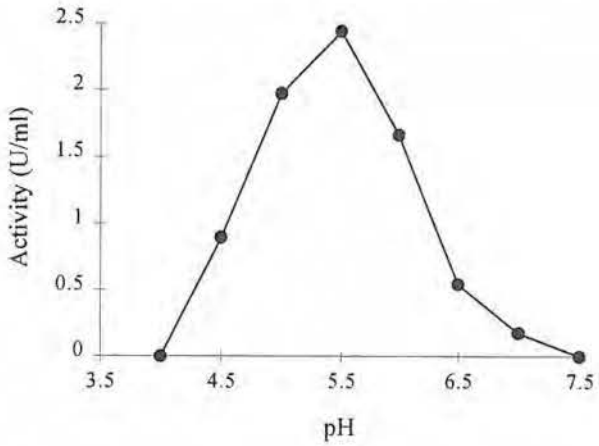

Fig. 2-12 pH optimum of PGE measured on a tributyrin emulsion

2.3.4.2 Interfacial denaturation

The activity of purified PGE was to a large extent dependent on the presence of BSA in the reaction medium. When purified PGE was assayed on a tributyrin emulsion without BSA, a rapid loss of activity occurred. On the contrary, optimal activity was reached and maintained for a considerable length of time, when the medium was supplemented with 2 μM BSA before adding the enzyme. Addition of BSA to an emulsion in which the enzyme was already inactivated did not restore the action of PGE (data not shown). Obviously PGE is subject to irreversible interfacial denaturation, a phenomenon that also occurs with the gastric lipases (Gargouri et al., 1986). Previously, Gargouri showed that the

addition of certain amphiphiles (BSA, ovalbumin or β-lactoglobulin) was necessary for the detection of enzyme activity in purified gastric lipases. With crude calf PGE extracts this surface inactivation was not observed as it was probably prevented by the presence of other proteins.

2.3.5 Influence of E600 and THL

E600 is a serine enzyme inhibitor binding covalently to serine residues. With 0.48 mM E600 a rapid and complete inhibition of PGE was observed after 45 min of incubation (Fig. 2-13 A).

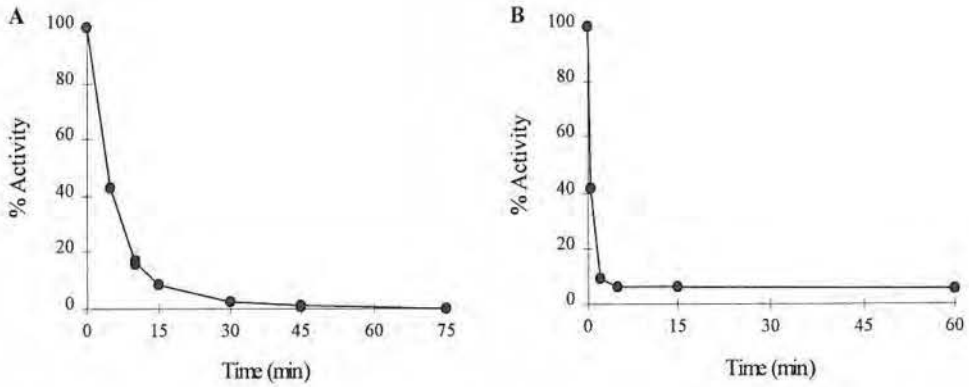

Fig. 2-13 Time course of the inactivation of PGE during incubation with E600 (A) and THL (B).
The inhibitors were added in a molar excess of 120 and 100 respectively. Residual PGE activity was measured in duplicate with tributyrin as a substrate.

This is in agreement with observations made with other lipases and confirms that a serine residue is important for lipolytic activity in lipases. A conserved Gly-Xaa-Ser-Xaa-Gly pentapeptide sequence is indeed present in all lipases sequenced so far. X-ray diffraction analysis of crystalline lipase from *Rhizomucor miehei* (Brady et al., 1990) and human pancreas lipase (Winkler et al., 1990) predicted a

central role for the serine residue of this conserved sequence in the Ser/His/Asp triad that is present in the catalytic site of these enzymes. When the enzyme is not attached to an oil/water interface this putative hydrolytic site is covered by a surface loop and therefore inaccessible. Crystal structure analysis from *Rhizomucor miehei* lipase inhibited by n-hexylphosphonate (Brozozowski et al., 1991) and E600 (Derewenda et al., 1992) showed a conformational change by which the conserved serine was exposed. It was proposed that the lipase-inhibitor complex is equivalent to the activated state that is generated when the enzyme binds to an oil/water interface. HGL and RGL, when inactivated by E600, lose their activity on both water-soluble and emulsified tributyrin but are still able to bind to lipid/water interfaces (Moreau et al., 1991). This provides strong evidence that the essential serine residue is involved in catalysis and not in lipid binding.

THL is a derivative of lipstatin, which is produced by *Streptomyces toxytricini*. THL was a generous gift of Dr. H. Lengsfeld (Hoffman La Roche, Basle, Switzerland). It has been demonstrated that THL binds to the essential serine residue of human and porcine pancreatic lipase (Peng et al., 1991; Guidoni et al., 1981). THL is described as a potent inhibitor of the lipases (Borgström 1988; Gargouri et al., 1991) and our data show that THL also inhibits PGE. Although inhibition of PGE by THL is almost immediate, a residual activity of about 5% remains, even after prolonged incubation (Fig. 2-13 B).

The inhibition of PGE by E600 and THL is in agreement with the assertion that a serine residue is necessary for the lipolytic activity of lipases in general.

2.3.6 Influence of thiol reagents and β-mercaptoethanol

DTNB and 4-PDS are classical thiol reagents. $C_{12:0}$-TNB a hydrophobic analogue of DTNB was a generous gift from Prof. R. Verger (CNRS, Marseilles). Sulfhydryl reagents and heavy metal ions like Hg^{2+} block free cysteine residues and are therefore used to investigate the influence of these residues on enzyme activity. Incubation of PGE with DTNB or 4-PDS, even for more than three hours, did not decrease the enzyme activity (Fig. 2-14 A and B), while $C_{12:0}$-TNB partially inhibited PGE (Fig. 2-14 C). This inhibition was less and much slower

than the one described for gastric lipases (Gargouri et al., 1991). Therefore the influence of the free fatty acid $C_{12:0}$ was tested on the enzyme activity. The inhibition pattern of $C_{12:0}$ was similar to that of $C_{12:0}$-TNB: 50% inhibition occurred after 10 min and 15 min respectively (Fig. 2-14 D). PGE was not inactivated with 33 mM Hg^{2+} (data not shown).

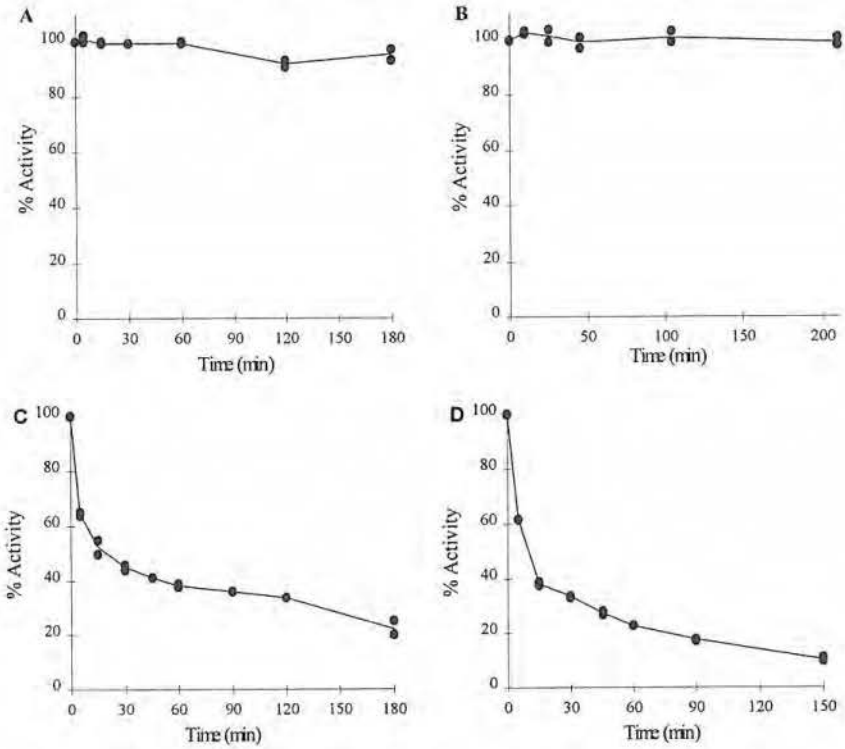

Fig. 2-14 Influence of thiol reagents on PGE activity

Purified PGE was incubated with a 100 molar excess of 4-PDS (A), DTNB (B), $C_{12:0}$-TNB (C), $C_{12:0}$ (D).

Residual PGE activity was determined by measuring tributyrin hydrolysis in the pH-stat at different incubation times. Data represent results of two independent experiments.

β-mercaptoethanol gave a definite but incomplete inhibition of PGE (Fig. 2-15), with about 20% of the enzyme remaining active.

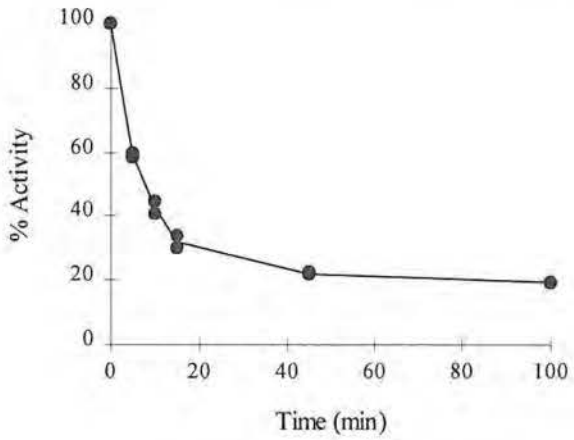

Fig. 2-15 Inhibition of PGE with β-mercaptoethanol

$Q_{FAST\ FLOW}$ purified PGE was incubated with 56 mM β-mercapthoethanol and residual activity was determined on a tributyrin emulsion.

In contrast to the gastric lipases, PGE is not inhibited by the sulfhydryl reagents DTNB and 4-PDS. In the gastric lipases three cysteine residues are conserved. After inhibition by sulfhydryl reagents, only one out of these three cysteines was titrated per enzyme molecule for HGL, RGL and DGL (Gargouri et al., 1988; Moreau et al., 1988; Carrière et al., 1991). The thiol modified gastric lipases could still penetrate into lipid monolayers but they were unable to hydrolyse their substrate. From the inhibition of the gastric lipases by DTNB, 4-PDS and $C_{12:0}$-TNB it was assumed that these enzymes possessed a sulfhydryl group that is either directly or indirectly involved in the catalytic mechanism (Gargouri et al., 1989). Therefore they were called 'sulfhydryl enzymes'. It was proposed that modification of this sulfhydryl group prevents the access of the substrate to the catalytic site. The fact that PGE is not inhibited by DTNB or 4-

PDS indicates that no free sulfhydryl group is involved in its activity. In contrast to this, PGE is only partially inhibited by the hydrophobic DTNB analogue $C_{12:0}$-TNB. We could show that this inhibition is due to the $C_{12:0}$ moiety since the same degree of inhibition was observed with the free fatty acid $C_{12:0}$.

Based on the inhibition of PGE with β-mercaptoethanol the presence of a disulphide bridge, important for the lipolytic activity can be postulated.

From our study it is clear that, contrary to the gastric lipases, no sulfhydryl group is involved in the catalytic mechanism of PGE. Accordingly, the term sulfhydryl enzyme does not apply to PGE (Timmermans et al., 1996)

2.3.7 NH_2-terminal sequence analysis of tryptic fragments of PGE

Purified PGE was subjected to mild cleavage with trypsin. Immunoblot analysis of the tryptic digest revealed the presence of three peptide bands under reducing conditions, P1, P2 and P3 (Fig. 2-16). The NH2-terminal sequences of P1 and P2 were identical: Ile-Ala-Lys-Asn-Pro-Glu-Ala-Ser-Met-Asn-Val-Ser-Gln-Met-Ile-Ser-Tyr. For P3 the sequence Glu-Thr-Leu-Asp-Val-Leu was found. These amino acid sequences fully correspond to those deduced from the nucleotide sequence of the cDNA encoding PGE (section 3.3.2). Under non-reducing conditions only peptide P1 was found on the immunoblot, indicating that the peptides P2 and P3 are linked by a disulphide bridge (Timmermans et al., 1996).

NH_2-terminal sequence analysis of native PGE was not possible, apparently because of a blocked amino terminus. This observation is contrary to the finding of Bernbäck et al., (1985) who determined Phe-Leu(Ile)-Gly as the NH_2-terminal tripeptide in his preparation of PGE. The reason for this result remains unclear.

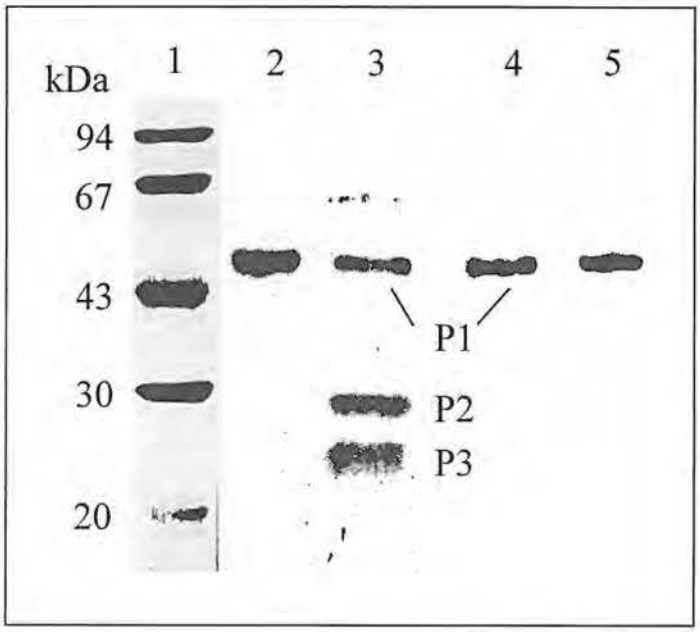

Fig. 2-16 Immunoblot analysis of PGE fragments obtained after trypsin digestion.

Lane 1: molecular weight standards.

Lane 2: native PGE under reducing conditions.

Lane 3: trypsin digested PGE under reducing conditions.

Lane 4: trypsin digested PGE under non-reducing conditions.

Lane 5: native PGE under non-reducing conditions.

PGE was detected with pAb's against PGE (1:1000). Visualisation was performed with swine anti-rabbit-AP (1:1000) and BCIP/NBT as the substrate.

2.4 Summary

An original purification procedure for calf PGE has been developed. Purified PGE is a 50 kDa glycoprotein. The enzyme has a pH optimum of 5.5 and is sensitive to interfacial denaturation.

PGE is inhibited by E600 and THL, suggesting that PGE is a serine enzyme. This is in agreement with the proposed catalytic triad Ser/His/Asp, common to all lipases.

PGE is quite distinct from the gastric lipases with regard to the involvement of a sulfhydryl group in its catalytic activity. On the basis of our results it is proposed that there is no sulfhydryl group involved in the catalytic mechanism of PGE and accordingly the term sulfhydryl enzyme should not apply to PGE.

3. Molecular cloning and nucleotide sequence of the calf pregastric esterase cDNA

3.1 Introduction

At the start of this work, only the cDNA's for HGL (Bodmer et al., 1987) and RLL (Docherty et al., 1985) had been cloned and sequenced. These enzymes showed a high sequence identity both at the nucleotide and the amino acid level. This made it quite reasonable to expect a comparably high degree of homology between PGE and these two lipases. Advantage was taken of this possibility in oligonucleotide primer design. PCR was used to amplify the cDNA encoding PGE from a cDNA library, prepared from calf tongue tissue, with primers based on the nucleotide sequence of highly conserved regions in HGL and RLL

3.2 Experimental procedures

3.2.1 Isolation of total RNA

Purification of total RNA was done as described by Chomczynki and Sacchi (1987).To eliminate RNase activity, all buffers were treated with 0.1 % DEPC (diethylpyrocarbonate) and the glassware was heated overnight at 180°C.

A calf tongue was obtained from a local slaughterhouse. The papillae vallatae were aseptically removed from the posterior dorsal region of the tongue and immediately frozen in liquid nitrogen. Approximately 5 g of the frozen tissue was homogenised (Polytron PT 1200, Kinematica) in 50 ml denaturation buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.1 M β-mercapto-ethanol, 0.5 % sarkosyl). This homogenate was mixed with 5 ml 2 M sodium acetate pH 4.0, 50 ml water-saturated phenol, 10 ml chloroform-isoamyl alcohol (49:1;v/v) and centrifuged. The RNA in the upper water phase was precipitated

with 50 ml isopropanol. The RNA pellet was dissolved in 15 ml denaturation buffer and again precipitated with isopropanol. The final RNA pellet was washed with 75 % ethanol and resuspended in 2.5 ml water.

The concentration of the isolated RNA was determined by measuring the optical density of the solution at 260 nm (1 OD_{260}= 40 µg/ml RNA) and the purity was estimated by measuring the ratio OD_{260}/OD_{280}. The integrity of the total RNA was checked by electrophoresis in a 1% agarose/formaldehyde gel (section 3.2.3).

3.2.2 Purification of poly(A)$^+$RNA

Poly(A)$^+$RNA was purified by affinity chromatography according to Aviv and Leder (1972). All manipulations were performed under RNase-free conditions. A 0.5 ml column of oligo(dT) cellulose (Clontech) was equilibrated with high-salt buffer (10 mM Tris pH 7.4, 1 mM EDTA, 0.5 M NaCl). Total RNA (2.5 ml) was mixed with sample buffer (10 mM Tris pH 7.4, 1 mM EDTA, 6 M NaCl) to a final concentration of 0.5 M NaCl and poured onto the oligo(dT) column. The polyadenylated fraction was allowed to bind and the liquid running through was reapplied two times. The column was washed with 1 ml high-salt and 1 ml low-salt buffer (10 mM Tris pH 7.4, 1 mM EDTA, 0.1 M NaCl). Finally the poly(A)$^+$RNA was eluted with 1 ml prewarmed elution buffer (10 mM Tris pH 7.4, 1 mM EDTA, 65°C). The eluted poly(A)$^+$RNA was precipitated by the addition of sodium acetate to a final concentration of 0.3 M and 2.5 volumes of ethanol. This solution was left overnight at -20°C and the next day, the precipitated poly(A)$^+$RNA was dissolved in 100 µl water. The purity, the concentration and the integrity of the poly(A)$^+$RNA was determined as described in section 3.2.1.

3.2.3 RNA electrophoresis

Purity and integrity of RNA and poly(A)$^+$RNA were controlled by submarine electrophoresis on a 1 % agarose/formaldehyde gel in 1x MOPS buffer (10x MOPS: 4.18% MOPS, 49.8 mM sodium acetate, 10 mM EDTA). A sample of the RNA (about 6 µg) was first denatured by heating for 15 min at 55°C in

1x MOPS with 6% formaldehyde and 50% formamide. Next, sample buffer (5x sample buffer: 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue) was added and the gel was run at a constant voltage (50V). The RNA was visualised by soaking the gel for 45 min in an ethidiumbromide solution (10 µg/ml).

3.2.4 Preparation of a calf tongue cDNA library

For the preparation of a cDNA library the Timesaver cDNA kit (Pharmacia) was used (Fig 3-1). The cDNA synthesis was monitored by the incorporation of radioactive nucleotides (α-^{32}P)dCTP. From the amount of precipitated radioactivity, the yield of first- and second-strand cDNA was calculated.

First-strand synthesis was carried out with MMLV reverse transcriptase. The reaction was initiated by a *Not*I/oligo(dT) primer having a *Not*I restriction site and an oligo(dT) tract that primes at the 3'-end of the poly(A)$^+$RNA's. Following first-strand synthesis, the RNA strand of the RNA:DNA hybrid was nicked with RNase H. Finally the nicked RNA was replaced with DNA by nick translation with DNA polymerase I. This resulted in double stranded cDNA. Ligation of an *Eco*RI adaptor to each end of the cDNA was followed by digestion with *Not*I. The *Not*I/*Eco*RI cDNA was directionally ligated in the *lacZ'* gene of the lambda ExCell *Not*I/*Eco*RI/CIP vector (Pharmacia). The recombinant lambda DNA was packaged *in vitro* using a lambda packaging extract (Boehringer Mannheim). Finally *E. coli* NM522 bacteria were infected with a fraction of the phage suspension.

An overnight culture of *E. coli* in LB medium (1% Bacto Tryptone, 0.5% Yeast Extract, 0.2% NaCl) was diluted 1/100 in LB medium with 0.2% maltose and grown to an OD_{600} of 1. The bacterial cells were resuspended in 1/4 volume of 10 mM $MgSO_4.7H_2O$, which resulted in an OD_{600} of 2. Different phage dilutions (1/10; 1/100; 1/1000) were prepared in SM buffer (0.1 M NaCl, 0.2% $MgSO_4.7H_2O$, 50 mM Tris, 0.01% gelatine; pH 7.5) and incubated for 20 min at 37°C with 100 µl of bacteria. After phage absorption, top agar (LB medium with 0.7% agar) with IPTG (0.65 mM) and X-gal (0.125%) was added and the bacteria were plated on LB plates (LB medium with 1.5% agar). After an overnight

incubation at 37°C the white and blue plaques were counted. From these values phage titer and cloning efficiency were calculated.

Because of the instability of the packaged phage particles the remainder of the phage suspension was amplified the next day. For this purpose 0.25 ml bacteria were incubated with 10^5 phages for 20 min at 37 °C and plated on LB plates. After growing for 7 hours at 37 °C the phages were eluted overnight at 4 °C by covering the plates with 10 ml SM buffer. Chloroform (0.5 ml) was added to the pooled phage lysates and they were stored at 4 °C. A part of the amplified library was also stored at -70 °C in the presence of 7% DMSO.

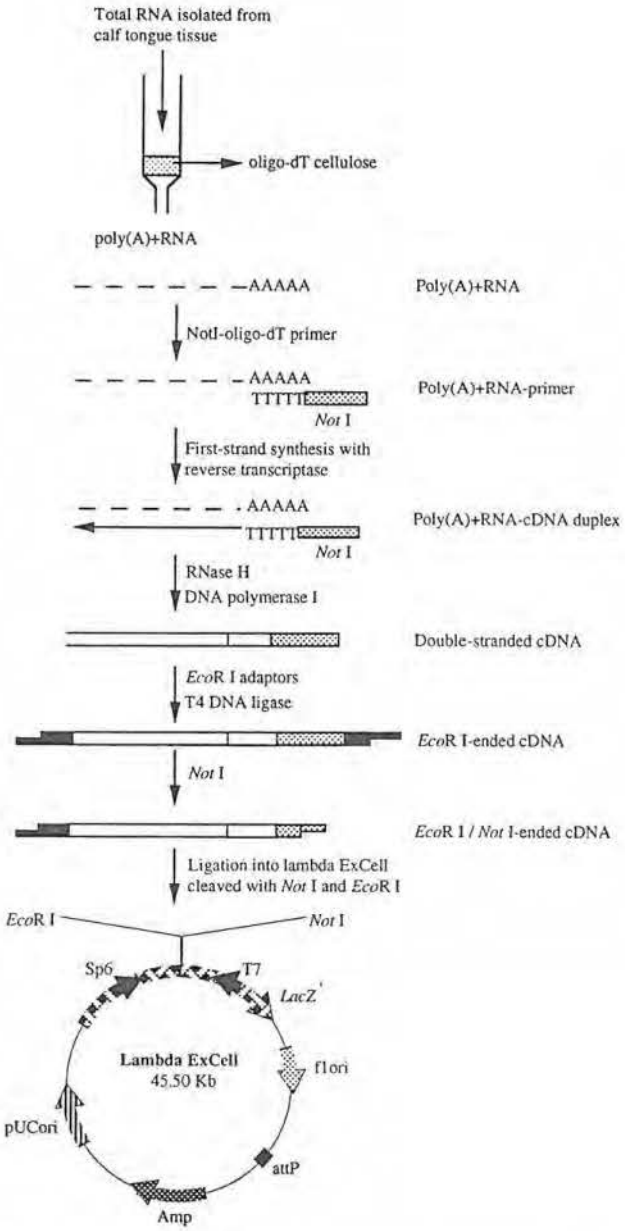

Fig. 3-1 Schematic representation of the procedure used for cDNA synthesis.

3.2.5 Polymerase chain reaction (PCR)

PCR (Saiki et al., 1988) is a rapid procedure for the enzymatic amplification of specific DNA segments. This technique was used to amplify parts of the PGE cDNA from the cDNA library.

The reaction mixture contained 20 pmol of 2 specific primers, 0.1 µg phage DNA, 0.2 mM dNTP, 10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatine and 2.5 Units Taq polymerase (Gibco BRL) in a total volume of 100 µl. This reaction mixture was overlaid with 100 µl mineral oil. After a first denaturation step (5 min at 94 °C) the mixture was subjected to 35 reaction cycles. Each cycle consisted of 1 min denaturation at 94 °C, 1 min annealing (the temperature of the annealing reaction was adapted to the T_m of the primers) and 2 min extension at 72 °C. The PCR reaction was terminated by a final extension step of 10 min at 72 °C. The amplified products were identified by electrophoresis on an appropriate agarose gel (section 3.2.9).

The oligonucleotide primers used in the PCR reactions were synthesised by Pharmacia (see sections 3.3.2, 4.2.1 and 5.2.1). All PCR reactions were performed in a Techne PHC-3 apparatus (New Brunswick Scientific).

3.2.6 Sequence analysis of the PCR products

For sequencing purposes, the PCR fragments were first cloned into the *lacZ'* gene of a pUC18 *Sma*I vector with the aid of the Sure Clone ligation kit (Pharmacia). During the PCR procedure, Taq polymerase adds a single dAMP to the 3' ends of the template by a template-independent polymerisation activity. This poses a problem in cloning PCR products. With the Sure Clone kit, PCR fragments are prepared for blunt-end ligation by utilising the 3'-5' exonuclease activity of the Klenow fragment of DNA polymerase I to remove these single-base 3' overhangs. After transformation of competent *E. coli* NM522 a few recombinant (white) colonies were chosen from the plates, and grown. Plasmid DNA was purified from the bacterial cultures and used for sequencing (section 3.2.8.2).

Sequence analysis was performed with the T7 sequencing system of Pharmacia. This kit is based on the dideoxy sequencing method of Sanger (Sanger

et al., 1977). Dideoxy sequencing depends on base-specific termination of enzyme-catalysed primer-extension reactions. Four separate reaction mixtures are prepared, all containing primers, enzyme, template DNA and the four deoxynucleotides, but each including a different chain-terminating dideoxynucleotide. The base at the 3' end of the chain-terminated fragments represents the base in the dideoxynucleotide used in each of the reaction mixtures.

In the T7 sequencing kit, primer extension is catalysed by T7 DNA polymerase instead of the Klenow fragment of DNA polymerase I used in the original procedure. Primer extension reactions are performed in two stages, a labelling and a termination reaction. The sequencing reactions were carried out as described in the manual supplied with the kit. In the labelling reaction (α^{35}S)dATP was used. For priming the universal M13 primer or the primers used for PCR were employed.

The samples were separated on a denaturating 6% polyacrylamide gel (6% acrylamide, 0.25% N,N'-methylenebisacrylamide, 42% urea, 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, 0.025% ammonium persulfate, 0.1% TEMED pH 8.3). The gels were run in a vertical sequencing apparatus (Biorad) at 2000 V and 150 mA. To increase the amount of information two samples of each reaction mixture were loaded on the same gel, with a three hours interval between the loadings. After fixation with 2% glacial acid, the gel was transferred to a filter-paper support and vacuum dried (45 min at 80°C) in a vacuum gel dryer (model 583, Biorad). The sequence was visualised by overnight exposure of the gel to Hyperfilm β-Max (Amersham) at room temperature.

Sequence results were analysed with the DNASIS software (Pharmacia).

3.2.7 Transformation of *E. coli*

Competent bacteria of the *E. coli* strains NM522 and Top10F' were prepared by the $CaCl_2$ method. An overnight bacterial cell culture was 1/100 diluted and grown to an OD_{600} of 0.375. After centrifugation the cell pellet was resuspended in 1/40 volume ice-cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% glycerol, 10 mM Pipes pH 7) and incubated for 30 min. After a second centrifugation the

cell pellet was again resuspended in $CaCl_2$ solution (1/200 of the original volume of the cell culture) and incubated overnight at 4 °C. The competent cells were stored in 100 µl fractions at -70 °C. For transformation, competent cells were mixed with plasmid DNA (10 ng) and subjected to a heat shock, which allows the DNA to enter the cells. The heat shock was performed by placing the mixture on ice for 10 min, followed by an incubation for two min at 42 °C. Finally the cells were grown in non-selective LB medium for one hour and plated on ampicillin (100 µg/ml) containing LB plates to allow identification of plasmid containing colonies.

3.2.8 DNA purification

3.2.8.1 Preparation of phage lambda DNA

Lambda phage DNA was purified with the Qiagen kit. DNA was prepared from plate lysates (section 3.2.4). These lysates were treated with chloroform (1 µl/ml) and centrifuged to remove bacteria and agarose. The supernatant was used to purify the phage DNA by anion exchange chromatography as described in the directions supplied with the kit. The DNA concentration was determined spectrophotometrically (1 OD_{260}= 50 µg/ml DNA).

3.2.8.2 Preparation of plasmid DNA

Small amounts of plasmid DNA were prepared from 5 ml bacterial cultures with Wizard minipreps (Promega). For larger plasmid preparations, 100 ml cultures were treated with the Qiagen plasmid midi kit. Both procedures are based on the alkaline lysis method of Birnboim et al. (1983). A small fraction of the DNA was always checked for purity by electrophoresis on a 0.6% agarose gel (section 3.2.9). The DNA obtained was used for sequencing and transformation experiments.

3.2.9 Electrophoresis of DNA and extraction of DNA from the agarose gels

The DNA fragments were separated by electrophoresis on agarose gels. The amount of agarose in the gel was adapted to the size of the fragments. After addition of loading buffer (10x loading buffer: 20% Ficoll 400, 0.1 M EDTA pH 8, 1% SDS, 0.25% bromophenol blue) the DNA samples were loaded onto the gels. A 100 bp ladder (Pharmacia) served as molecular weight marker. The gels were run in a submarine system with TBE buffer (0.6 M Tris-borate, 0.2 M EDTA) at a constant voltage of 40 V.

DNA fragments were removed from the gels with the Qiaex gel extraction kit. With this system the agarose is dissolved and the DNA is selectively adsorbed to Qiaex silicagel particles in the presence of high salt. The elution is done with a low salt buffer.

3.3 Results and discussion

3.3.1 Construction of calf tongue cDNA library

About 1.7 mg total RNA (OD_{260}/OD_{280}= 2.04) was obtained from 5 g of tongue tissue. The integrity of the extracted RNA was confirmed on a 1% agarose/formaldehyde gel. The appearance of the 18S and the 28S ribosomal RNA bands served as an indicator for the integrity (Fig. 3-2).

Poly(A)$^+$RNA was isolated from the total RNA by affinity chromatography on an oligo(dT) cellulose column. This resulted in 22 μg of poly(A)$^+$RNA (OD_{260}/OD_{280}= 1.92) or 1.3% of the total RNA.

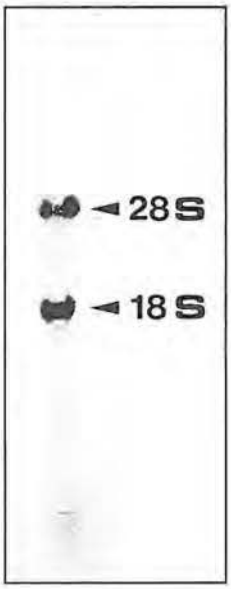

Fig. 3-2 1% agarose/formaldehyde gel electrophoresis of 6 µg of total RNA, isolated from calf tongue.

About 5 µg poly(A)$^+$RNA was used for the cDNA synthesis. This cDNA was directionally cloned in the *lacZ'* gene of lambda ExCell DNA and after *in vitro* packaging used to infect *E. coli* NM522. From the ratio of white and blue plaques a cloning efficiency of 1.37 x 10^7 pfu/µg cDNA was calculated. After amplification of the cDNA library in *E. coli* NM522, phage DNA was purified and analysed by PCR for the presence of calf PGE sequences.

3.3.2 Screening of the calf tongue cDNA library by PCR and sequencing of the PCR products

The calf tongue cDNA library was screened for PGE sequences by PCR. Two primers (93L68, 93L69) were chosen based on the published sequences of RLL (Docherty et al., 1985) and HGL (Bodmer et al., 1987). The 93L68 primer corresponds to a region with 96% identity between RLL and HGL. The 93L69 primer corresponds to a region that is 88% identical. Each of these primers was

used in combination with the T7 primer, to amplify a specific fragment of PGE (Fig. 3-3).

The PCR products were analysed on a 1.5% agarose gel. Only with the 93L69 primer a PCR product of 860 base pairs was obtained, corresponding to the size expected from the RLL and HGL sequences (Fig. 3-4).

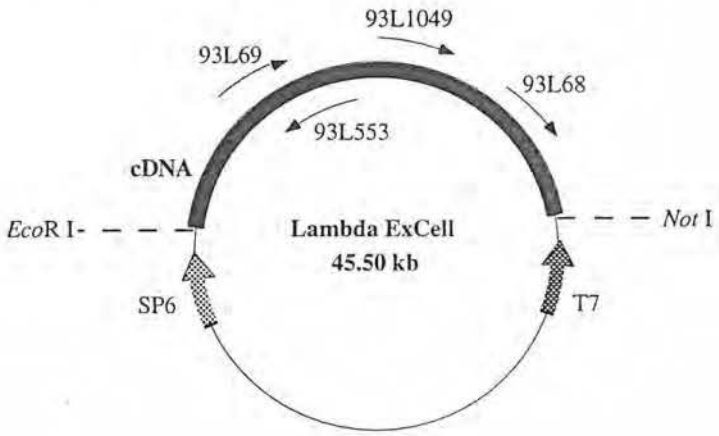

Fig. 3-3 Schematic representation of the priming sites of the primers used in the PCR reactions.
The sequences of the primers were as follows:

93L68: 5'-GACTTCATCTGGGCAATGGATGCC-3'

93L69: 5'-GTTGGTCATTCTCAGGGCACCACC-3'

93L1049: 5'-ATAGCACATAATCCAGCAGG-3'

93L553: 5'-AGCCAATGTGGGACTGGTAGA-3'

SP6: 5'-GATTTAGGTGACACTATAG-3'

T7: 5'-TTAATACGACTCACTAT-3'

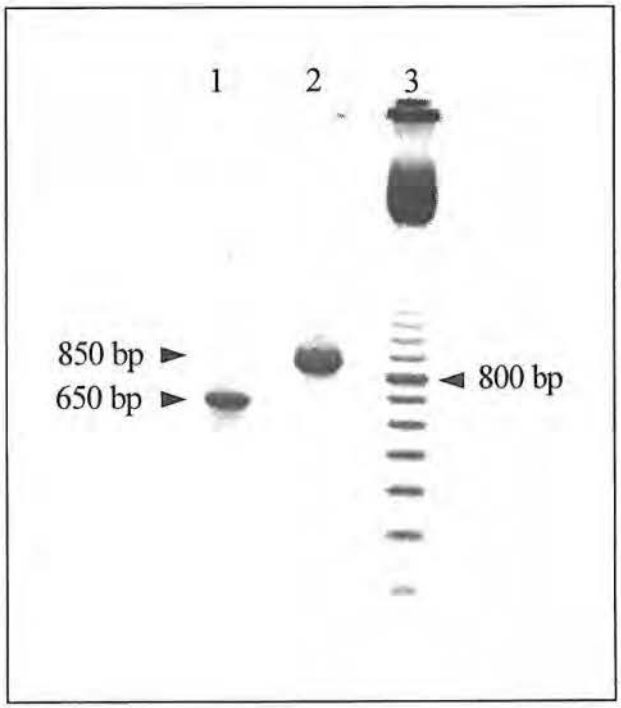

Fig. 3-4 PCR amplification of PGE fragments.

The PCR products were analysed on a 1.5% agarose gel.

Lane 1: PCR fragment of 650 base pairs amplified with primers SP6 and 93L553.

Lane 2: PCR fragment of 850 base pairs, amplified with primers T7 and 93L69.

Lane 3: 100 base pairs ladder marker.

For sequence analysis this fragment was extracted from the agarose gel and subcloned into the *lacZ'* gene of the pUC18 vector. Positive colonies were selected by blue/white screening. Cultures of the positive colonies were grown overnight. The plasmid DNA from the cells was purified and compared with pUC18 DNA without the insert by electrophoretic analysis on a 0.6% agarose gel. Clones containing the PCR fragment were sequenced with the 93L69, T7 and the universal M13 primer. Since it is not possible to sequence the entire 850 base pair

fragment in one operation with these primers it was necessary to synthesise an additional primer (93L1049).

To amplify the lacking 5'end of the PGE cDNA an antisense primer (93L553) based on the 850 base pair fragment already sequenced, was used in combination with the SP6 primer (Fig. 3-3). A PCR fragment of about 650 base pairs was amplified (Fig. 3-4). This second PCR product was also sequenced, after subcloning into pUC18.

This resulted in the entire coding sequence for PGE, shown in fig. 3-5 (Timmermans et al., 1994). From this sequence it is obvious that no amplification with primer 93L68 can be expected. Indeed this primer has a non-matching nucleotide at its 3'end.

```
CGG ACG AGG AGA GCT AGG TTT TTC ATT TAC CTT CAA GAA ACT AGA AGG CAT TCA CTT TGG  60
TGA CAG TTG AAA(ATG TGG TGG CTA CTT GTA ACA GTG TGT TTC ATC CAC ATG TCT GGA AAT 120
               ( M  W  W  L  L  V  T  V  C  F  I  H  M  S  G  N  -4
GCA TTT TGT)TTC CTT GGA AAA ATT GCT AAG AAC CCT GAA GCC AGT ATG AAT GTT AGT CAG 180
 A  F  C ) F  L  G  K  I  A  K  N  P  E  A  S  M  N  V  S  Q  17
ATG ATT TCC TAC TGG GGC TAC CCA AGT GAG ATG CAT AAA GTT ATA ACT GCG GAT GGT TAT 240
 M  I  S  Y  W  G  Y  P  S  E  M  H  K  V  I  T  A  D  G  Y  37
ATC CTT CAG GTC TAT CGG ATT CCT CAT GGA AAG AAT AAT GCT AAT CAT TTA GGT CAG AGA 300
 I  L  Q  V  Y  R  I  P  H  G  K  N  N  A  N  H  L  G  Q  R  57
CCT GTT GTG TTT CTG CAG CAT GGT CTT CTT GGA TCA GCC ACA AAC TGG ATT TCC AAC CTG 360
 P  V  V  F  L  Q  H  G  L  L  G  S  A  T  N  W  I  S  N  L  77
CCC AAG AAC AGC CTG GGC TTC CTC CTG GCA GAT GCT GGT TAT GAC GTG TGG CTG GGG AAC 420
 P  K  N  S  L  G  F  L  L  A  D  A  G  Y  D  V  W  L  G  N  97
AGC AGA GGA AAC ACC TGG GCC CAG GAA CAT TTA TAC TAT TCA CCA GAC TCC CCG GAA TTC 480
 S  R  G  N  T  W  A  Q  E  H  L  Y  Y  S  P  D  S  P  E  F  117
TGG GCT TTC AGC TTT GAT GAA ATG GCG GAA TAT GAC CTT CCA TCT ACA ATT GAT TTC ATC 540
 W  A  F  S  F  D  E  M  A  E  Y  D  L  P  S  T  I  D  F  I  137
TTA AGG AGA ACA GGA CAG AAG AAG CTA CAC TAT GTT GGC CAT TCC CAA GGC ACC ACC ATT 600
 L  R  R  T  G  Q  K  K  L  H  Y  V  G  H  S  Q  G  T  T  I  157
GGT TTT ATC GCC TTT TCT ACC AGT CCC ACA TTG GCT GAA AAA ATC AAA GTC TTC TAT GCA 660
 G  F  I  A  F  S  T  S  P  T  L  A  E  K  I  K  V  F  Y  A  177
TTA GCC CCA GTT GCC ACA GTG AAG TAC ACC AAG AGC CTG TTT AAC AAA CTT GCA CTT ATT 720
 L  A  P  V  A  T  V  K  Y  T  K  S  L  F  N  K  L  A  L  I  197
CCT CAC TTC CTC TTC AAG ATT ATA TTT GGT GAC AAA ATG TTC TAC CCA CAC ACT TTT TTG 780
 P  H  F  L  F  K  I  I  F  G  D  K  M  F  Y  P  H  T  F  L  217
GAA CAA TTT CTT GGT GTT GAA ATG TGC TCC CGT GAG ACA CTG GAT GTC CTT TGT AAG AAT 840
 E  Q  F  L  G  V  E  M  C  S  R  E  T  L  D  V  L  C  K  N  237
GCC TTG TTT GCC ATT ACT GGA GTT GAC AAT AAA AAC TTC AAC ATG AGT CGC TTA GAT GTG 900
 A  L  F  A  I  T  G  V  D  N  K  N  F  N  M  S  R  L  D  V  257
TAT ATA GCA CAT AAT CCA GCA GGA ACT TCT GTT CAA AAC ACC CTC CAC TGG AGA CAG GCT 960
 Y  I  A  H  N  P  A  G  T  S  V  Q  N  T  L  H  W  R  Q  A  277
GTT AAG TCT GGG AAA TTC CAA GCT TTT GAC TGG GGA GCC CCA TAT CAG AAC CTA ATG CAT 1020
 V  K  S  G  K  F  Q  A  F  D  W  G  A  P  Y  Q  N  L  M  H  297
TAT CAT CAG CCC ACA CCT CCC ATC TAC AAT TTA ACA GCC ATG AAT GTC CCA ATT GCA GTA 1080
 Y  H  Q  P  T  P  P  I  Y  N  L  T  A  M  N  V  P  I  A  V  317
TGG AGT GCT GAC AAT GAC CTG TTG GCT GAC CCT CAG GAT GTT GAC TTT CTG CTT TCA AAA 1140
 W  S  A  D  N  D  L  L  A  D  P  Q  D  V  D  F  L  L  S  K  337
CTC TCT AAT CTC ATT TAC CAC AAG GAA ATT CCA AAT TAC AAT CAC TTG GAC TTT ATC TGG 1200
 L  S  N  L  I  Y  H  K  E  I  P  N  Y  N  H  L  D  F  I  W  357
GCA ATG GAT GCA CCT CAA GAA GTT TAC AAT GAA ATT GTT TCT TTG ATG GCC GAA GAC AAA 1260
 A  M  D  A  P  Q  E  V  Y  N  E  I  V  S  L  M  A  E  D  K  377
AAG TAG TTC TGG ATT TAG AGA ATT TTT CAT TTG CTT TTT CCA AAA TAG TTT CTT CTC GCT 1320
 K  *                                                          378
TAC ATG ATT TCT GTA CTG TTT GAA ATG CAA TGC TTC TTT CTG TAA TGT TGA CTT TCA AAA 1380
TAT ATT AGC ATC AAC AAA AAA AAA AAA AAA AAA  14113
```

Fig. 3-5 Nucleotide sequence of PGE cDNA and deduced amino acid sequence. The termination codon TAG is marked by an asterisk. The signal peptide of 19 amino acids is between brackets. A putative polyadenylation signal (AAATATA, in italics) is present 11 nucleotides upstream from the polyA site. The red amino acids indicate the conserved sequence containing the active site serine residue. Three possible glycosylated asparagine residues are represented in green. Two cysteine residue are coloured in blue. The GenBank accession No is L26319.

3.3.3 Analysis of PGE cDNA and comparison with other mammalian lipases

The complete cDNA sequence and the corresponding deduced amino acid sequence of PGE is shown in fig. 3-5. PGE contains an open reading frame of 1200 nucleotides with the first Met in frame at position 73 and a stop codon at position 1264. This corresponds to a coding capacity of 397 amino acids. The location of the NH_2-terminal H_2N-Phe-Leu(Ile)-Gly (Bernbäck et al., 1985) sequence, leads to the conclusion that PGE originally contains a 19 amino acid signal peptide. The mature enzyme is a 378 amino acid protein with a calculated molecular mass of 42,960 Da. As already mentioned in chapter 2 the purified calf PGE is a glycoprotein with a molecular mass of 50 kDa. The difference with the calculated molecular mass is due to the carbohydrate component. The calculated molecular mass corresponds with the one obtained after PGE deglycosylation (section 2.3.3). HGL, DGL, RGL and RLL have similar molecular masses.

The NH_2-terminal amino acid sequences of the fragments of PGE, determined by Edman degradation (section 2.3.7) are identical to those deduced from the cDNA sequence and clearly show that the purified enzyme corresponds to the protein encoded by the cDNA sequence of PGE. The deduced amino acid composition of PGE and that obtained from amino acid analysis of the purified enzyme, as determined by Bernbäck et al. (1985) are compared in table 3-1. With the exception of Trp, which in the latter case was not determined, the compositions are almost entirely consistent.

As can be seen from table 3-2 there is a high nucleotide and amino acid identity between PGE, RLL (Docherty et al., 1985), HGL (Bodmer et al., 1987), RGL and DGL (Bénicourt et al., 1993). For optimal alignment a gap of three nucleotides in the PGE cDNA must be introduced at positions 142-144. At the nucleotide level PGE shows 82% identity to HGL and 76% to RLL. The amino acid sequence of PGE shows 75% identity to HGL, 73% to DGL, 73% to RGL and 71% to RLL. The degree of conservation in the signal peptide sequences is much lower. Furthermore PGE, like the other preduodenal lipases shows a close identity

with lysosomal lipase, which confirms the existence of an acid lipase gene family (Anderson and Sando, 1991; Ameis et al., 1994).

Table 3-1 Amino acid composition of PGE, deduced from the cDNA sequence and obtained from amino acid analysis of the purified enzyme[a]

amino acid	from cDNA sequence	from amino acid analysis[b]
Val	5.6	5.7
Leu	10.5	10.1
Ile	5.8	5.6
Ser	6.4	5.9
Thr	4.8	4.7
Gly	5.6	7.4
Ala	7.9	9.1
Cys	0.5	1.0
Met	2.9	2.7
Asx	11.9	12.4
Glx	7.8	7.9
Arg	2.1	2.5
Lys	5.8	4.5
His	4.0	3.7
Phe	6.1	6.0
Tyr	5.0	3.7
Trp	2.4	n.d.*
Pro	5.6	6.5

[a] *amino acid composition is expressed in mole percent*

[b] *results from Bernbäck et al. 1985*

n.d.: not determined

Table 3-2 Percentage identity between PGE, RLL[a], DGL[b], RGL[b], HGL[c] and HLAL[d], based on the deduced amino acid sequences

	PGE	RLL	DGL	RGL	HGL	HLAL
PGE	100	71	73	73	75	56
RLL		100	77	79	78	58
DGL			100	81	85	59
RGL				100	84	58
HGL					100	59
HLAL						100

[a] *sequence data from Docherty et al., 1985*

[b] *sequence data from Bénicourt et al., 1993*

[c] *sequence data from Bodmer et al., 1987*

[d] *sequence data from Ameis et al., 1994*

Sequence alignment of PGE with the other preduodenal lipases shows the presence of an additional amino acid after residue number 4, which explains the shift in amino acid numbering (Fig. 3-6). In the amino acid sequence of PGE three potential N-glycosylation sites are present (Asn^{14}, Asn^{251} and Asn^{307}). These sites are highly conserved in the other preduodenal lipases with the exception of Asn^{308} which is absent in RLL. In addition to the three glycosylation sites, the gastric lipases and RLL contain a glycosylation site at Asn^{80} (Fig. 3-6).

All gastric lipases (HGL, DGL and RGL) have three conserved cysteine residues (Cys^{227}, Cys^{236} and Cys^{244}). Instead, PGE has only two of these conserved cysteines (Cys^{226} and Cys^{235}) while RLL contains a fourth cysteine residue (Cys^{27}).

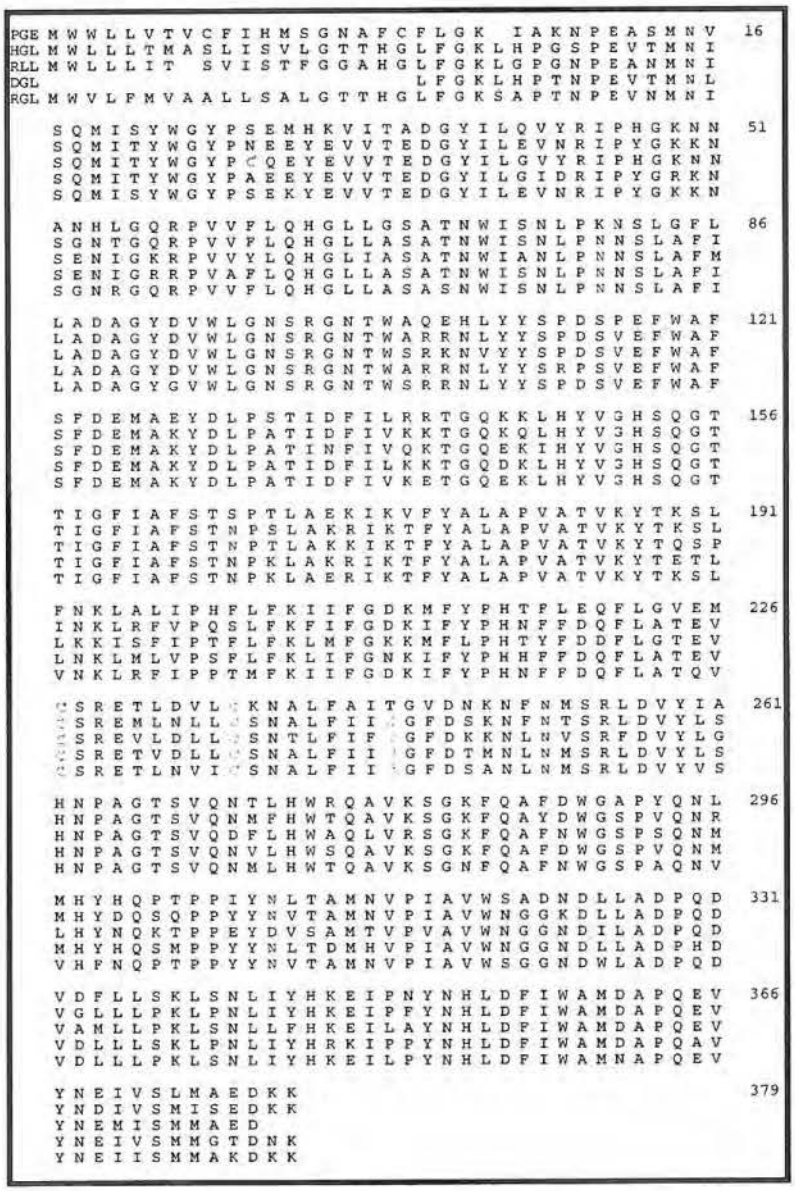

Fig. 3-6 Amino acid sequence alignment of preduodenal lipases

For optimal amino acid sequence alignment of PGE with the other preduodenal lipases a gap of 1 amino acid was introduced after amino acid residue 4. Cysteine residues are coloured in blue and possible N-glycosylation sites are represented in green.

In the gastric lipases Cys^{227} and Cys^{236} are separated by an Arg^{229}. For HGL and RGL the fragments generated after tryptic cleavage at Arg^{229} are separated under both reducing and non-reducing conditions. This indicates that Cys^{236} and Cys^{244} are involved in a disulphide bridge, implicating that Cys^{227} is free (Aoubala et al., 1994). Blocking this free cysteine inactivates the gastric lipases (Gargouri et al., 1988; Moreau et al., 1988; Carrière et al., 1991). PGE contains only two of these conserved cysteines and is not inhibited by sulfhydryl reagents (section 2.3.6). On the contrary it is inhibited by β-mercaptoethanol. This leads to the assumption that in PGE the conserved residues form a disulphide bridge. Comparison of the NH_2-terminal sequences determined by Edman degradation of tryptic PGE fragments (section 2.3.7), with the deduced amino acid sequence indicates trypsin cleavage sites at Lys^4 and Arg^{228} (Fig. 3-7). The fragments P2 and P3, generated by tryptic cleavage at Arg^{228} were not separated in the absence of a reducing agent (section 2.3.7). This is in contrast to what was found for RGL and HGL, and clearly indicates that in PGE there is a disulphide bridge between Cys^{226} and Cys^{235}, linking P2 and P3 (Timmermans et al., 1996).

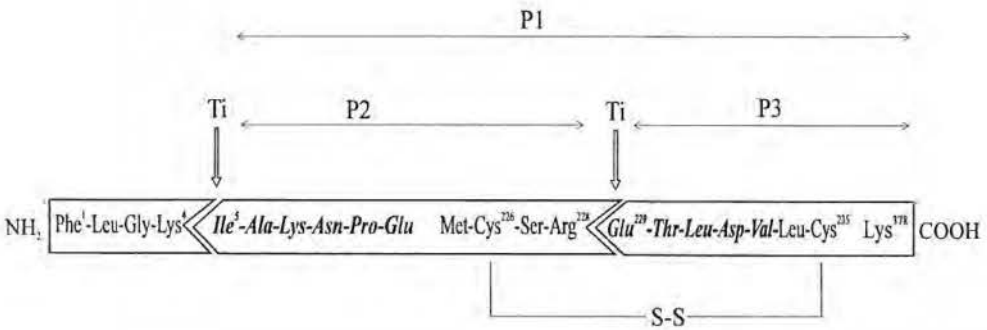

Fig. 3-7 Schematic representation of the amino acid sequence of the peptide fragments obtained after digestion of PGE with trypsin.
Amino acids in bold italics were determined by Edman degradation. Trypsin cleavage sites (Ti), tryptic fragments P1, P2 and P3 and the disulphide bridge are indicated

In this respect it is interesting to note that RLL has four cysteine residues. Since these may be involved in the formation of two disulphide bridges it is possible that RLL has no free sulfhydryl group as in the case of PGE. Along this line, preduodenal lipases can be classified in two categories. The first category includes the pregastric lipases which are not secreted by the stomach and do not possess a free thiol group. The second category are the gastric lipases. These enzymes are produced in the stomach and have a functional free sulfhydryl group that plays an important role in their catalytic activity. Unfortunately, inhibition experiments as described in this study on PGE, are not yet available for RLL.

PGE shows no sequence identity with non-acid lipases, except for a conserved Gly-Xaa-Ser-Xaa-Gly sequence which is found in all lipases sequenced until now (Kordel et al., 1991).

From X-ray diffraction studies on human pancreatic lipase (Winkler et al., 1990) and *Rhizomucor Miehei* lipase (Brady et al., 1990) it was deduced that the serine residue in a Ser/His/Asp triad plays a key role in the catalytic process. The arrangement of amino acids in the catalytic triad is similar to the one in serine proteases like trypsin and chymotrypsin (Brady et al., 1990). Moreover, the Gly-Xaa-Ser-Xaa-Gly sequence in lipases surrounding the active site serine is also conserved in serine proteases (Table 3-3).

Table 3-3 Sequence around the essential serine residue in lipases and other serine enzymes

Trypsin	Gly-Asp **Ser**-Gly-Gly	
Chymotrypsin	Gly-Asp-**Ser**-Gly-Gly	
Trombin	Gly-Asp-**Ser**-Gly-Gly	
Elastase	Gly-Asp-**Ser**- Gly-Gly	
Factor Xa	Gly-Asp-**Ser**-Gly-Gly	
Human PL	Gly-His-**Ser**^{152}-Leu-Gly	
Porc PL	Gly-His-**Ser**^{152}-Leu-Gly	
Dog PL	Gly-His-**Ser**^{152}-Leu-Gly	
Horse PL	Gly-His-**Ser**^{152}-Leu-Gly	
Human LPL	Gly-Tyr-**Ser**^{132}-Leu-Gly	
Bovine LPL	Gly-Tyr-**Ser**^{132}-Leu-Gly	
Human HL	Gly-Tyr-**Ser**^{147}-Leu-Gly	
Rat HL	Gly-Tyr-**Ser**^{147}-Leu-Gly	
HGL	Gly-Asn-**Ser**^{99}-Arg-Gly	Gly-His-**Ser**^{153}-Gln-Gly
RGL	Gly-Asn-**Ser**^{99}-Arg-Gly	Gly-His-**Ser**^{153}-Gln-Gly
DGL	Gly-Asn-**Ser**^{99}-Arg-Gly	Gly-His-**Ser**^{153}-Gln-Gly
RLL	Gly-Asn-**Ser**^{99}-Arg-Gly	Gly-His-**Ser**^{153}-Gln-Gly
PGE	Gly-Asn-**Ser**^{98}-Arg-Gly	Gly-His-**Ser**^{152}-Gln-Gly

It has to be noticed that PGE, just like the other preduodenal lipases, accommodates two of these conserved sequences, specifically Gly-Asn-**Ser**^{98}-Arg-Gly and Gly-His-**Ser**^{152}-Gln-Gly. In section 2.3.5 it was shown that a serine residue is important for lipolytic activity of PGE.

From the known lipase sequences the following consensus pattern for the Gly-Xaa-Ser-Xaa-Gly region can be deduced:

[Leu/Ile/Val]-Xaa-[Leu/Ile/Val/Phe/Tyr]-[Leu/Ile/Val/Ser/Thr]-Gly-[His/Tyr/Trp/Val]-**Ser**-Xaa-Gly-[Gly/Ser/Thr/Ala/Cys].

For PGE the amino acid sequence around **Ser**^{152} is in conformity with the consensus pattern, indicating that **Ser**^{152} and not **Ser**^{98} is most probably involved in the catalytic triad. There is at present no indication where the two other amino acids (His and Asp), involved in the formation of the catalytic triad, are located since information on the three-dimensional structure of preduodenal lipases is not yet available.

A search in the Genbank revealed 1400 occurrences of the Gly-Xaa-Ser-Xaa-Gly pentapeptide. However most of the proteins concerned do not exhibit a hydrolytic activity. The question remains whether this pentapeptide sequence is evidence for an evolutionary link between the lipases and the serine proteases (Derewenda et al., 1991).

3.4 Summary

The deduced amino acid sequence of the cDNA encoding calf PGE reveals that the mature enzyme is a protein of 378 amino acids with a calculated molecular mass of 42,960 Da.

PGE shows a high degree of identity with the other preduodenal lipases, both at the nucleotide and the amino acid sequence level. Comparison of the amino acid sequence of PGE with non-preduodenal lipases reveals little identity, except for the sequence around the essential serine residue that takes part in the formation of the assumed catalytic triad.

4. Expression of the pregastric esterase cDNA in *E. coli*.

4.1 Introduction

Bacterial expression of the pregastric esterase cDNA in *E. coli* was performed with a pGEX plasmid (Pharmacia). This vector allows the intracellular expression of heterologous proteins as fusion proteins with *Schistosoma japonicum* glutathione-S-transferase (GST) (Smith and Johnson, 1988). The pGEX plasmid also provides an inducible *tac* promoter and a factor Xa protease recognition site for the cleavage of the protein from GST.

4.2 Experimental procedures

4.2.1 Cloning of PGE cDNA in pGEX-5X-2

The cDNA sequence encoding PGE was amplified by PCR. Phage DNA from the cDNA library was used as a template, with two primers flanking the PGE cDNA sequence. PGE cDNA was amplified with and without its signal peptide sequence. Each primer (Fig. 4-1) contained an internal restriction site and was chosen so that the cDNA encoding PGE could be cloned directionally in the correct open reading frame. The PCR reaction was performed as described in section 3.2.5 with an annealing temperature of 50°C.

A

Forward: 5' GTGACAGTCGACAATGTGGTG 3'
*Sal*I
Backward: 5' CTCTAACTCGAGAACTACTT 3'
*Xho*I

B

Forward: 5' TGTTGGATCCACATGTCTGGA 3'
*Bam*HI
Backward: 5' CTCTAACTCGAGAACTACTT 3'
*Xho*I

Fig. 4-1 Nucleotide sequence of the primers used for the amplification of PGE cDNA by PCR

A: primers used to amplify PGE cDNA, with the signal peptide sequence

B: primers used to amplify PGE cDNA, without the signal peptide sequence

The PCR products were examined by electrophoresis in an agarose gel and purified with the Wizard PCR preps DNA purification system (Promega). The purified PCR product and the pGEX-5X-2 vector (Fig. 4-2) were digested for 1h at 37°C with 2U of the appropriate restriction enzymes per µg DNA. For cloning the PGE cDNA with the signal peptide sequence, the PCR product and the vector were cut with *Sal*I and *Xho*I restriction enzymes. For cloning the PGE cDNA without the signal peptide sequence, the PCR product and the vector were restricted with *Bam*HI and *Xho*I. In both cases, the digested vector was dephosphorylated by incubation for 30 min at 37°C with 1.4 U of calf intestinal phosphatase (CIP) per 2 µg vector. Restriction enzymes and CIP were purchased from Pharmacia and used according to the accompanying instructions.

The restricted PCR fragment and vector were recovered from agarose gels (section 3.2.9) and incubated in a 5/1 molar ratio with T4 DNA ligase (2 hours, 16°C) as described by Sambrook et al., (1989). *E. coli* NM522 cells were transformed with the ligation mixture (section 3.2.7). Transformants were grown in 10 ml cultures and the plasmid DNA was purified as described in section 3.2.8.2.

The presence of a PGE cDNA insert was determined by electrophoretic analysis of restriction digests of the isolated plasmid DNA. The enzymes used for these digestions were the same as those used for cloning.

Transformants for which the presence of PGE cDNA was established in this way were used for expression studies. *E. coli* cells, both non-transformed and transformed with wild type vector DNA served as controls.

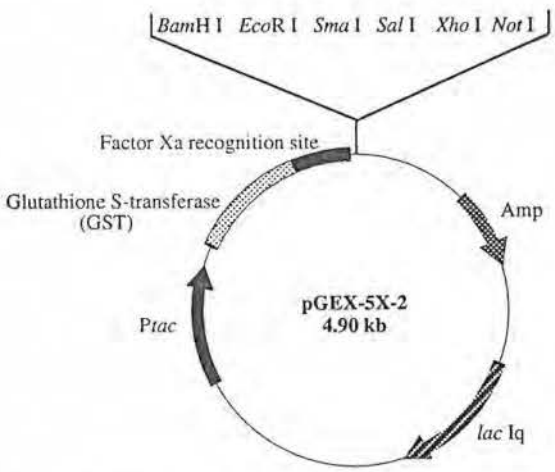

Fig. 4-2 Vector pGEX 5X-2

4.2.2 Screening of pGEX recombinants for fusion protein expression

Several transformants containing PGE cDNA were grown at 37°C for 4 hours in 2 ml 2 x YT-G medium (1.6% Tryptone, 1% yeast extract, 0,5% NaCl,

2% glucose, pH 7) with ampicillin (100 µg/ml) to an OD_{600} of 0.6-0.8. To induce the production of the fusion protein 20 µl 100 mM IPTG was added and the cultures were incubated for an additional two hours. The cells were collected by centrifugation and the pellet was resuspended in 300 µl PBS. The cell suspension was lysed by sonication on ice (45 sec. at 6 µ) and centrifuged (12000 g) for 10 min at 4°C. The fusion protein in the soluble fraction was purified by affinity chromatography. The supernatant was first incubated for 5 min with 20 µl of a 50% slurry of gluthatione-agarose (Sigma G4510) and centrifuged. After three washes with PBS the fusion protein was eluted by the addition of 10 µl of reduced glutathione (10 mM in 50 mM Tris-HCl pH 8). The eluate was analysed by SDS-PAGE (section 2.2.3) and Western blotting (section 2.2.5.1). Primary screening for fusion protein was performed with goat anti-GST antiserum (Pharmacia, 1/200). For further identification, pAb's (1/1000) and mAb 3C9C7 (1/2000) directed against purified PGE were used.

Transformants, producing the fusion protein were grown and induced on a larger scale. For that purpose 100 ml bacterial cultures were grown and induced with 0.1 mM IPTG for 4 hours at 37°C. After centrifugation the bacterial cells were resuspended in 5 ml PBS and sonicated (see above). The supernatant obtained after centrifugation of the sonicated cells was incubated with 100 µl 50% gluthatione-agarose slurry and the fusion protein was eluted with 50 µl reduced gluthatione solution. In order to optimise the yield of fusion protein several growth conditions were tested: growth at 30°C instead of 37°C after induction, lower IPTG concentration (< 0.1 mM) for induction and shorter induction times (2 and 3 hours).

4.2.3 Solubilisation of inclusion bodies

Pellets from sonicated 100 ml bacterial cultures (section 4.2.2) were used to purify inclusion bodies (Marston and Hartley, 1990). The pellets were washed with 10 ml 0.5% Triton X-100. To extract the fusion protein from the precipitated dense bodies, they were resuspended in 4 ml of different denaturing solutions: 2 M, 6 M and 8 M urea; 2 M and 6 M guanidinium-HCl; 0.25% Sarkosyl; 1 M

NaSCN. All these denaturant solutions contained 0.1 mM PMSF. Renaturation was performed by overnight dialysis against PBS. Undissolved material was removed by centrifugation.

4.2.4 Factor Xa Cleavage of the fusion protein

The pGEX-5X-2 vector contains a cleavage site for the blood coagulation factor Xa protease. This allows an easy removal of the GST moiety from the fusion protein. Factor Xa cleaves after the Arg residue in the Ile-Glu-Gly-Arg sequence.

The GST part of the fusion protein was removed by treating 25 µl affinity purified fusion protein with 15 µg factor Xa (Boerhinger) overnight at room temperature.

4.2.5 Measurement of PGE activity

Activity of expressed PGE was either determined colorimetrically with PNPB (section 2.2.1.2) or by a radioactive method, using tri(^{14}C)oleoylglyceride (Dupont) as the substrate.

The radioactive emulsion was prepared as follows: 1 ml trioleoylglyceride (20 mg/ml toluene) and 0.58 ml tri(^{14}C)oleoylglyceride (0.05 mCi/25 ml toluene) were mixed. Toluene was evaporated under a stream of nitrogen and the triacylglycerols were washed 3 times with 0.5 ml heptane. After evaporation of the heptane 3.3 ml gum arabic solution (5% gum arabic in 0.2 M citrate phosphate buffer pH 5) was added and the suspension was sonicated for 4 min. Finally 2 ml BSA (10% BSA in 0.2 M citrate phosphate buffer pH 5) and 2.7 ml water were added.

For testing the enzyme activity, 10 µl samples were incubated for 1 hour at 37°C with 490 µl of the emulsion. The reaction was stopped by the addition of 3.25 ml methanol/chloroform/heptane (1.41:1.25:1) and 0.75 ml borate/carbonate buffer (0.14 M, pH 10.5). The mixture was shaken and centrifuged (10 min, 4000 rpm). The upper organic phase was mixed with 5 ml scintillation fluid

(Ultima Gold LSC cocktail; Packard) and its radioactivity was measured in a liquid scintillation counter (Wallac 1410; Pharmacia).

4.3 Results and discussion

4.3.1 Expression and purification of GST-PGE

After amplification by PCR (Fig. 4-3), the PGE cDNA was cloned into the pGEX-5X-2 vector. *E. coli* NM522 were transformed both with wild type vector and with vector containing the PGE cDNA. The presence of the PGE cDNA sequence in some transformants was determined by restriction enzyme analysis (Fig 4-4).

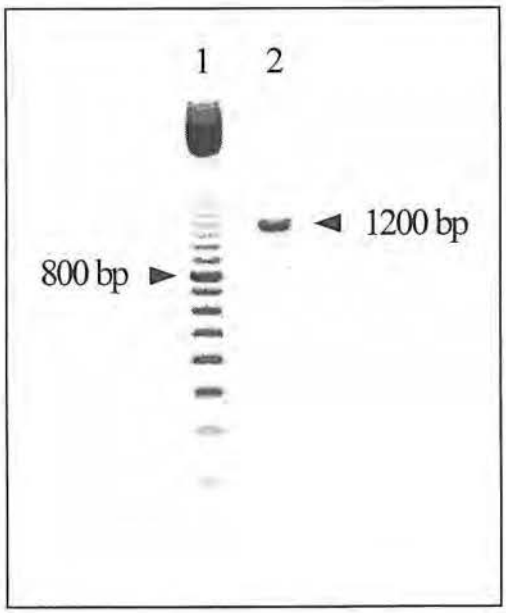

Fig. 4-3 Electrophoretic analysis of the PCR amplified PGE cDNA.
Electrophoresis was carried out on a 1% agarose gel.
Lane 1: 100 bp ladder marker
Lane 2: amplified cDNA for PGE with its signal peptide sequence

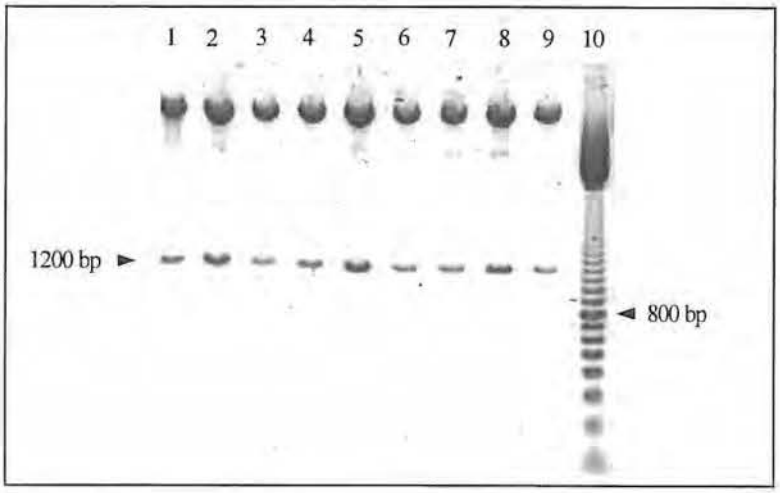

Fig. 4-4 Examination of the presence of the PGE cDNA insert in transformants by restriction enzyme analysis.
Lane 1-9: restricted plasmid DNA isolated from E. coli NM522 transformants
Lane 10: 100 bp ladder

Primary screening for fusion protein expression was done on 2 ml cultures of wild type and PGE transformants. After induction they were checked for the presence of GST or GST-PGE respectively. A culture of non-transformed bacteria was always included as a control. The supernatant of the sonicated cells was subjected to affinity chromatography with a glutathione-agarose slurry and examined. With SDS-PAGE no difference in protein band patterns was observed between non-transformed bacteria and bacteria transformed with wild type vector or with vector containing PGE cDNA. Immunoblotting with goat anti-GST antiserum and with anti-PGE antibodies (pAb's and mAb 3C9C7), however, showed a distinct protein band in 80% of the PGE cDNA containing transformants which was absent in wild-type transformants and in the non-transformed control cells.

Two transformants (A9 and B5) and a wild type transformant were grown in 100 ml cultures. Transformant A9 contains PGE cDNA with the signal peptide and

transformant B5 contains PGE cDNA without the signal peptide. In both transformants analysis of the affinity purified fusion protein on SDS-PAGE showed a band with an apparent molecular mass of 64 kDa which is not present in the supernatant of the wild type transformant. Only a few contaminant *E. coli* proteins were copurified by this procedure. Western blot analysis with anti-GST (Fig. 4-5) and anti-PGE antisera confirmed that the 64 kDa band is GST-PGE.

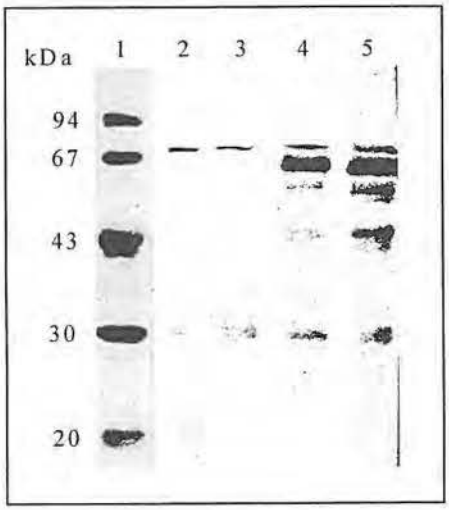

Fig. 4-5 Western blot analysis of glutathione-agarose treated cell extracts of non-transformed E. coli NM522, wild type and PGE transformants (A9 and B5).
Lane 1: molecular weight markers
Lane 2: non-transformed E. coli
Lane 3: wild type transformant
Lane 4: PGE transformant A9
Lane 5: PGE transformant B5
GST-PGE was detected with a goat anti-GST serum, diluted 1/200. Peroxidase conjugated anti-goat-HRP antibodies (1/1000) in combination with DAB were used for colour development.

4.3.2 Cleavage with Factor Xa

In the pGEX-5X-2 expression vector a specific protease cleavage site for blood clotting factor Xa is incorporated directly behind the GST moiety. Proteins of the supernatants from a sonicated wild type transformant and from PGE transformant A9 were purified by glutathione-agarose treatment and incubated overnight with factor Xa. Both Xa-treated and untreated samples were analysed by immunoblotting (Fig. 4-6). Identical results were obtained with pAb's and with mAb 3C9C7. Xa cleavage of GST-PGE was incomplete because uncleaved fusion protein was still present after an overnight incubation. After cleavage of the GST moiety a new peptide band with a molecular mass of 42 kDa was detected. Taking in account that GST has a molecular mass of 26 kDa this is in good agreement with the expected molecular mass for the PGE portion.

The molecular mass calculated from the deduced amino acid sequence is 42.9 kDa (section 3.3.3) whereas a value of 40 kDa was found on SDS-PAGE of the unglycosylated form of purified PGE (section 2.3.3).

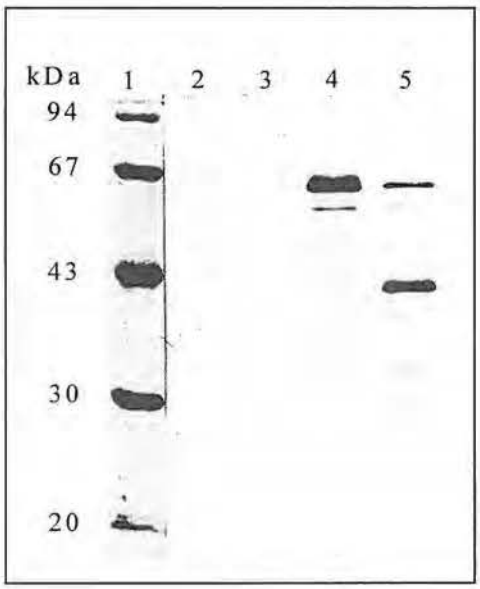

Fig. 4-6 Western blotting of GST-PGE before and after treatment with blood clotting factor Xa.

Lane 1: molecular weight marker

Lane 2 and 3: affinity purified GST from a wild type transformant before (lane 2) and after (lane 3) digestion with Xa

Lane 4 and 5: affinity purified GST-PGE from PGE transformant A9 before (lane 4) and after (lane 5) treatment with factor Xa

PGE was detected with PGE pAb's, diluted 1/1000. For colour development anti-rabbit-HRP antibodies (1/1000) were used, with DAB as a substrate.

4.3.3 Solubilisation of inclusion bodies

The production of recombinant proteins in bacteria is limited by the formation of cytoplasmic aggregates, called inclusion bodies (Schein, 1989). To reduce the formation of inclusion bodies different growth conditions were tested (section 4.2.2). Although there was a remarkable increase in soluble fusion protein

when the growth temperature was lowered to 30°C after induction, the major portion remained insoluble.

The pellet from sonicated bacteria, containing the insoluble GST-PGE was solubilised with several denaturing agents: urea, guanidinium-HCl, sarkosyl and NaSCN. The denaturant was removed by dialysis against PBS and the unsolubilised material was removed by centrifugation. Only the solubilised material obtained after treatment with 6 M and 8 M urea showed a clear band on SDS-PAGE representing GST-PGE (Fig. 4-7).

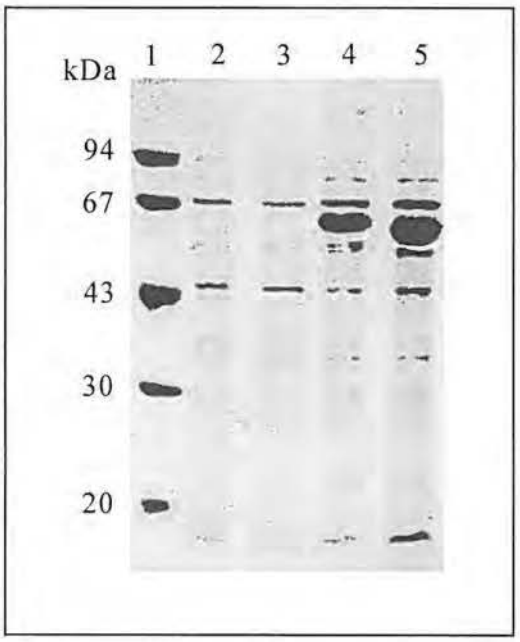

Fig. 4-7 SDS-PAGE of GST-PGE solubilised from inclusion bodies with 8 M urea

Lane 1: molecular weight markers.
Lane 2: non-transformed E. coli
Lane 3: wild type transformant
Lane 4: PGE transformant A9
Lane 5: PGE transformant B5

4.3.4 PGE activity

Affinity purified GST-PGE and GST-PGE solubilised from inclusion bodies were examined for PGE activity. Neither the purified nor the solubilised GST-PGE was active with PNPB or tri(^{14}C)oleoylglyceride as the substrate. Furthermore it was also impossible to detect any activity after cleaving off the GST moiety.

In section 2.3.3 it was shown that PGE is a glycoprotein and that deglycosylation greatly decreased its activity. The inability of *E.coli* to carry out glycosylation may be one of the reasons why the expressed GST-PGE is inactive.

4.4 Summary

Bacterial expression of PGE as a GST fusion protein resulted in the production of a 64 kDa protein. Neither the fusion protein nor the PGE obtained after cleaving off the GST moiety showed any enzyme activity.

5. Expression of the pregastric esterase cDNA in *Pichia pastoris*

5.1 Introduction

In the previous chapter it was mentioned that native PGE is a glycosylated protein and that glycosylation is probably essential for catalytic activity. Consequently expression of the cDNA for PGE in a eukaryotic system could be a better way of producing enzymatically active PGE. As a first eukaryotic expression system, the yeast *Pichia pastoris* was chosen. *Pichia* presents all the conveniences of prokaryotic systems in terms of short generation times and ease of culture conditions. In addition proteins synthesised by *Pichia* are subjected to all posttranslational modifications known to occur in eukaryotic cells, including glycosylation. In contrast to *Saccharomyces cerevisiae,* no hyperglycosylation occurs in *Pichia.*

Pichia is a methylotrophic yeast, capable of using methanol as its sole carbon source. The first step in the metabolism of methanol is its oxidation to formaldehyde by the enzyme alcohol oxidase. *Pichia* contains two genes for alcohol oxidase: *AOX1* and *AOX2*. The *AOX1* gene is responsible for the major part of the alcohol oxidase activity and its expression is induced and regulated by methanol. The highly inducible promoter of the *AOX1* gene is used in the *Pichia* vectors to drive the expression of a foreign gene.

The *Pichia* expression system uses a *HIS4** (gene coding for histidinol dehydrogenase) mutant *Pichia* strain which is auxotrophic for histidine. Homologous recombination between the *AOX1* or the *HIS4** locus of the *Pichia* genome and the plasmid DNA, containing the PGE expression cassette and the wild type *HIS4* gene, generates His^+ transformants. Recombination at the *AOX1* locus can occur in two basic ways generating two different phenotypes of His^+ transformants: His^+ Mut^+ and His^+ Mut^S (slow methanol utilisation).

The **His^+ Mut^S** phenotype is due to the loss of all *AOX1* activity as the result of the complete removal of the *AOX1* coding region. This can be explained by a

gene **replacement** that arises from a double crossover between the 5' and 3' *AOX1* regions of the vector and the *Pichia* genome (Fig. 5-1). Due to the very low level of expression of the *AOX2* gene little alcohol oxidase activity remains present, which explains the slow methanol utilisation.

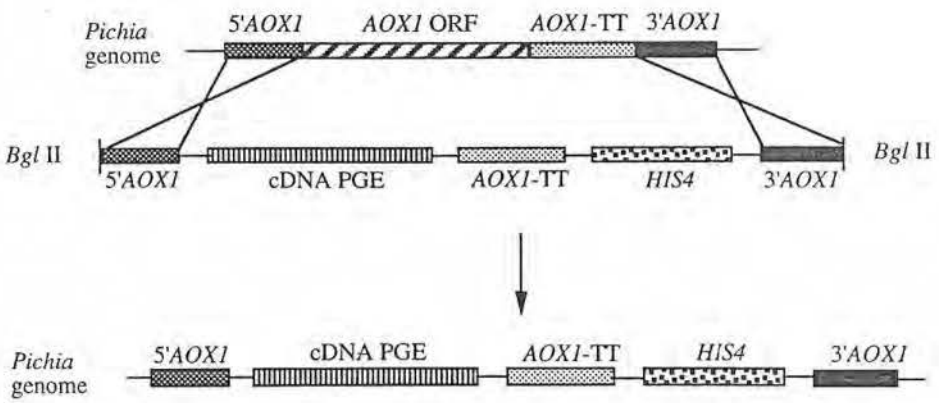

Fig. 5-1 Replacement of the AOX1 gene in the genome of Pichia with a PGE cDNA expression cassette by a double cross over.

The second phenotype (**His$^+$ Mut$^+$**) is caused by gene insertion at the *AOX1* or *HIS4** genes. These transformants are still able to use methanol at the normal rate. A single crossover between the *AOX1* sequences on the vector and the yeast genome or between the *HIS4** gene on the genome and the *HIS4* gene on the vector results in the addition of the gene of interest to the *Pichia* genome. In both cases the *AOX1* gene remains intact (Fig. 5-2 A and B).

Furthermore multi-copy integration can occur either at the *AOX1* and/or *HIS4** loci by a combination of independent recombination events. This can also result in His$^+$ Mut$^+$ and His$^+$ Mut^S phenotypes.

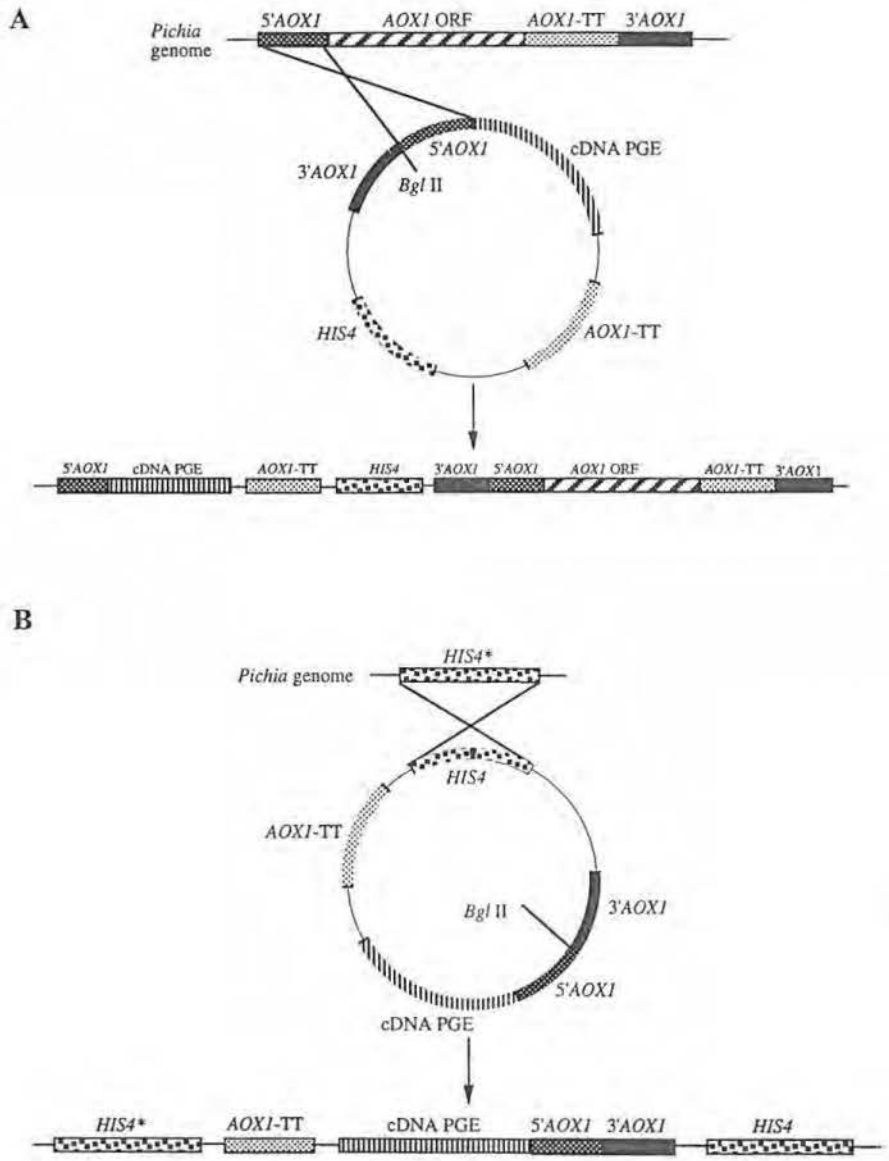

Fig. 5-2 Gene insertion events in the Pichia genome.

A: Insertion of the PGE cDNA expression cassette next to the AOX gene

B: Insertion of the PGE cDNA expression cassette next to the HIS4 gene*

5.2 Experimental procedures

5.2.1 Cloning of PGE cDNA in *Pichia* expression vectors

PGE cDNA without its signal peptide sequence, was amplified by PCR (section 3.2.5) and cloned in frame with the secretion signal peptide open reading frame of two vectors, pPIC9 and pHIL-S1(Fig. 5-3 and 5-4). These vectors contain different secretion signal peptide sequences: pHIL-S1 contains the signal sequence for *Pichia* acid phosphatase (PHO1) and pPIC9 contains the signal sequence for the alpha-mating factor pre-propeptide of *S. cerevisiae*. Both vectors carry an ampicillin resistance gene allowing selection in *E. coli* Top 10F' and the *HIS4* gene for selection in *Pichia* GS115, a *HIS4** mutant.

The vector constructs were made as described in section 4.2.1 and were used to transform competent *E. coli* TOP10F' (section 3.2.7). Plasmid DNA, isolated from the transformants was examined for the presence of a PGE insert by restriction enzyme analysis, and used for the transformation of *Pichia.*

A

Forward: 5' ATCCACAGGCCTGGAAATGCA 3'
*Stu*I
Backward: 5' AGCGGCCGCAAAATTCTCTAAATCCAG 3'
*Not*I

B

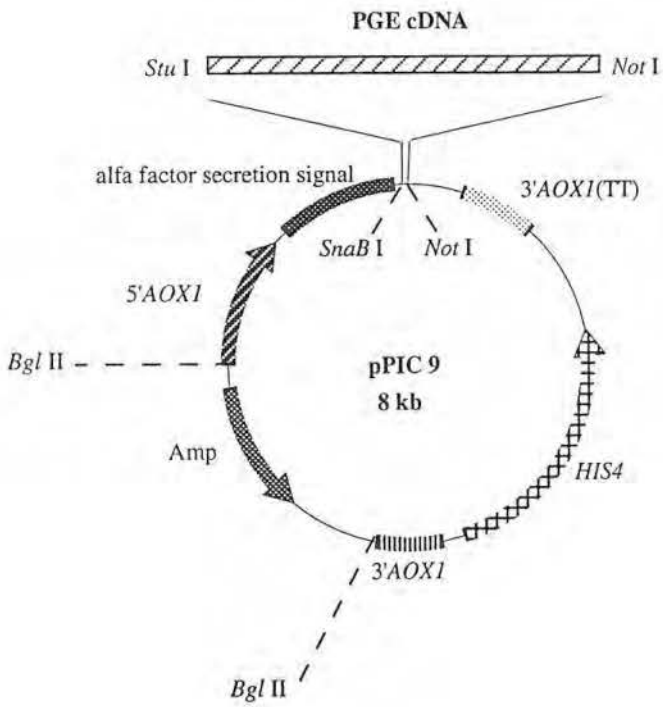

Fig. 5-3 Cloning of PGE cDNA in the pPIC9 vector

A: nucleotide sequence of primers used for amplification of PGE cDNA by PCR

B: pPIC9 vector

A

Forward: 5' ATCCACAGGCCTGGAAATGCA 3'

*Stu*I

Backward: 5' AATTCTCTGGATCCAGAACTACTT 3'

*Bam*HI

B

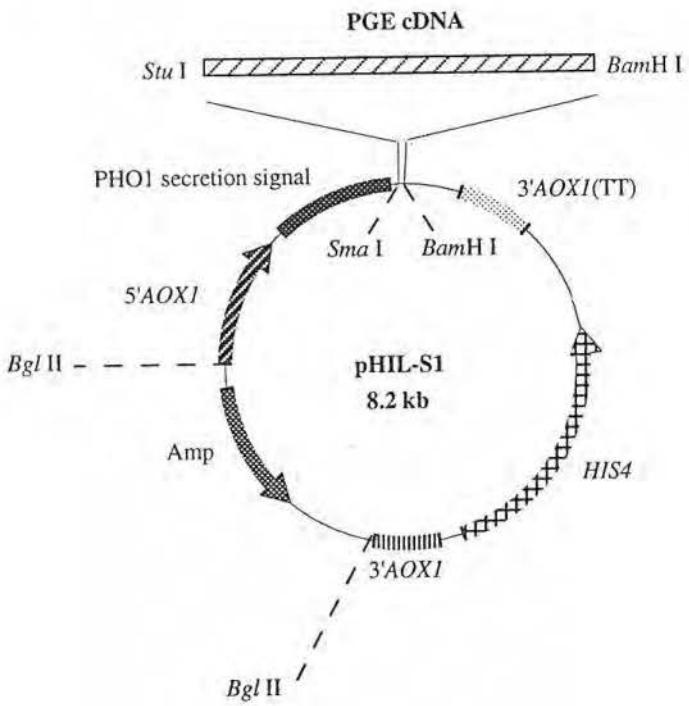

Fig. 5-4 Cloning of PGE cDNA in the pHIL S1 vector

A: nucleotide sequence of primers used for the amplification of PGE cDNA by PCR

B: pHIL-S1 vector

5.2.2 Transformation of *Pichia*

Pichia strain GS115 was used for transformation. This strain is defective in the histidinol dehydrogenase gene (*HIS4**) and consequently is unable to synthesise histidine.

To enable yeast to take up DNA, partial removal of the cell wall is necessary. For this purpose the spheroplast module of Invitrogen was used. This module is based on hydrolysis of the yeast cell wall by zymolase. Spheroplasts were prepared as described in the manual supplied with the module. The yeast spheroplasts were incubated with 10 µg plasmid DNA, cut with *Bgl*II just before the 5' *AOX1* promoter sequence and after the 3' *AOX1* sequence (see fig. 5-3 and fig. 5-4). Cutting the plasmid favours integration by homologous recombination at the *AOX1* locus in the *Pichia* genome.

The expression plasmids carry the *HIS4* gene which complements the defective *HIS4** gene in *Pichia* GS115. This allows the selection of transformants by their ability to grow on histidine-deficient medium. His^+ transformants were selected by plating the spheroplast-DNA suspension on Regeneration Dextrose medium (RD: 1 M sorbitol, 2% agar (Difco), 1.34% Yeast Nitrogen Base (Difco), 1% dextrose) without histidine.

5.2.3 PCR analysis of His^+ transformants

His^+ transformants were grown overnight in 10 ml YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C and the cell pellet was used for the isolation of genomic DNA. The pellet was washed with 500 µl water and resuspended in 200 µl breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH8, 1 mM EDTA pH 8). After the addition of 200 µl glass beads and 200 µl phenol/chloroform/isoamyl alcohol (25:24:1) the mixture was vigorously vortexed for 3 min. Finally 200 µl TE buffer was added and the mixture was centrifuged. The upper phase was drawn off and precipitated with 1 ml ethanol. After centrifugation the precipitate was dissolved in 400 µl TE buffer. 30 µl RNase A (1 mg/ml) was added and the solution was incubated for 5 min at 37°C. Finally the DNA was precipitated by the addition of 10 µl ammonium acetate

(4 M) and 1 ml ethanol. The pellet was dried and resuspended in 100 µl TE buffer. 2 µl of this DNA solution was used for PCR with primers complementary to the 5' *AOX1* promoter and the 3'*AOX1*-TT sequences (Fig. 5-5).

5' *AOX1* primer: 5' GACTGGTTCCAATTGACAAGC 3'

3' *AOX1* primer: 5' GCAAATGGCATTCTGACATCC 3'

Fig. 5-5 Nucleotide sequence of primers used to analyse His$^+$ transformants

The PCR mixture was boiled for 10 min and immediately placed on ice before adding Taq polymerase. The PCR reaction was simultaneously performed with GS115 *Pichia* genomic DNA as a control. PCR was carried out as described in section 3.2.5 at an annealing temperature of 50°C, and PCR products were analysed by electrophoresis on a 0.8% agarose gel.

5.2.4 RT-PCR analysis of *Pichia* transformants

Total RNA was extracted with acidic phenol and SDS at 65°C. Transformants were grown overnight at 30°C in 10 ml Minimal Glycerol medium (MGY: 1.34% Yeast Nitrogen Base (Difco), 1% glycerol). The cell suspension was centrifuged (1500 g, 5 min) The cell pellet was resuspended in 10 ml Minimal Methanol medium (MM: 1.34% Yeast Nitrogen Base, 0.5% methanol) and the cells were grown for an additional 2 days at 30°C. The cells were collected by centrifugation and the cell pellet (after brief washing with ice-cold water) was resuspended in 400 µl TES solution (0.5% SDS, 10 mM EDTA, 10 mM Tris pH 7.5). Next 400 µl acid phenol (pH 5) was added, the mixture was vigorously vortexed and incubated at 65°C with occasional vortexing. After 45 min the mixture was placed on ice for 5 min and centrifuged (10 min at 15000 g). The aqueous phase was transferred to a clean tube and 400 µl acid phenol was added.

The mixture was placed on ice for 5 min and centrifuged again. To precipitate the RNA, 40 µl 3 M sodium acetate pH 5.3 and 1 ml 100% ice-cold ethanol were added to the aqueous phase. The mixture was centrifuged, the RNA pellet was washed with 100 µl ice-cold 70% ethanol and centrifuged once more. Finally the RNA pellet was resuspended in 75 µl water and used for the purification of poly(A)$^+$ RNA as described in section 3.2.2.

RT-PCR was performed with the Superscript kit of Gibco BRL. Poly(A)$^+$RNA was copied to cDNA with an oligo-dT primer and reverse transcriptase (RT). The poly(A)$^+$RNA was degraded by RNase treatment and the cDNA was used as template for PCR (section 3.2.5) with two primers specific for PGE (Fig. 5-6). The amplified products were analysed by electrophoresis on a 1% agarose gel.

Forward: 5' TGAATTCTGTTTCCTTGGAAAAATTGC 3'

Backward: 5' AGCCAATGTGGGACTGGTAGA 3'

Fig. 5-6 Nucleotide sequence of primers used for RT-PCR

5.2.5 Expression and screening of *Pichia* transformants

Mut$^+$ transformants were grown in 25 ml cultures, and Mut^S transformants in 100 ml cultures. In both cases a single colony was inoculated in the appropriate volume of MGY medium. After an overnight growth at 30°C the cells were centrifuged and resuspended either in 100 ml (Mut$^+$ transformants) or in 20 ml (Mut^S transformants) MM medium, to induce expression. Induction was maintained by the addition of 0.1% methanol every 24 h. At different times (24h, 48h ,72h, 96h,....) a fraction of 1 ml of the cultures was removed and centrifuged. As controls two established *Pichia* transformants were always grown:

{HSA (His^+ Mut^S) and β-Gal (His^+ Mut^+)} simultaneously with the PGE transformants.

Both the culture medium and the cell contents were analysed for protein expression by SDS-PAGE (section 2.2.3) and Western blotting (section 2.2.5.1). The medium (1 ml) was precipitated by the addition of 100 µl sodium deoxycholate (0.15%) and 100 µl trichloroacetic acid (72%). The precipitate was resuspended in 30 µl 0.5 M Tris pH 8.5 and used for analysis.

Cells pelleted by centrifugation were broken up by the addition of 100 µl breaking buffer (50 mM sodium phosphate pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% glycerol) and an equal volume of acid washed glass beads. The mixture was briefly vortexed and placed on ice for 30 seconds. This cycle was repeated 8 times. The supernatant obtained after centrifugation of the cell lysate was analysed for the presence of PGE. Western blotting was performed with pAbs (1/1000) and mAb 3C9C7 (1/2000) against purified PGE.

5.3 Measurement of PGE activity

Activity of recombinant PGE in the culture medium and in the supernatant of the cell lysates was measured colorimetrically with the PNPB method (section 2.2.1.2). The medium was concentrated either by lyophylisation or by ultrafiltration (Centricon-30; Amicon) before measuring the activity.

Protein concentrations were determined by the BCA method (Pierce). Specific activity is expressed as OD_{405}/mg protein.

5.4 Results and discussion

5.4.1 Analysis of *Pichia* transformants

Pichia strain GS115 was transformed with pPIC9 and pHIL-S1 vectors containing PGE cDNA as an insert. Transformants were selected on RD plates without histidine. If homologous recombination occurred between the vector DNA and the *Pichia* genome, the *HIS4* wild type gene of the vector will complement the defective GS115 *HIS4** gene, resulting in His^+ *Pichia* transformants capable of growing without histidine. His^+ transformants generated with pPIC9 and pHIL-S1 are respectively called 8/x and 30/x. As explained in section 5.1 homologous recombination can happen in several ways creating two different His^+ phenotypes: His^+ Mut^S and His^+ Mut^+.

Analysis by PCR of the His^+ transformants allowed to screen for the presence of the PGE cDNA sequence in the *Pichia* genome. Furthermore the phenotype and the nature of the recombination event giving rise to it could be determined. In fig. 5-7 a typical result of such a PCR analysis is shown.

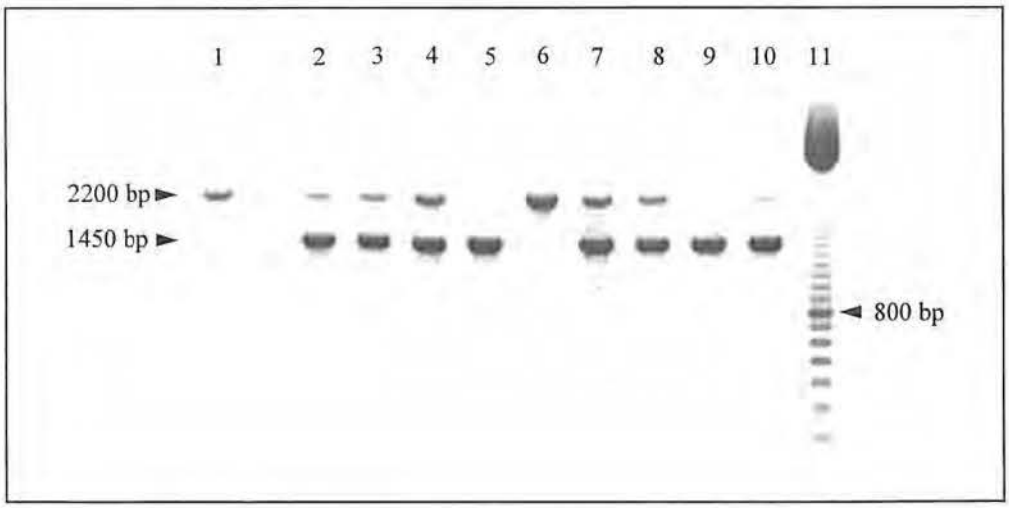

Fig. 5-7 PCR analysis of Pichia transformants generated with pHIL-S1

Three possibilities are distinguished:

- When no insertion occurs only one band of 2.2 kb corresponding to the *AOX1* gene is amplified (lanes 1 and 6 in fig. 5-7).

- When insertion has take place two bands are visible. One band corresponds to the *AOX1* gene (2.2 kb), the second originating from the inserted PGE sequence. The PGE cDNA itself is 1.2 kb long. To this, the size of the signal peptide sequence must be added: 450 base pairs in the case of the α-factor of pPIC9 and 240 base pairs for the PHO1 sequence of pHIL-S1. This results in a final size of 1650 base pairs for PGE with the α-factor (data not shown) and 1440 base pairs for PGE with the PHO1 signal peptide sequence (lanes 2,3,4,7,8 and 10 in fig. 5-7).

- When the *AOX1* gene is replaced by PGE, only one band is visible. The size of this band is 1650 base pairs for the clones transformed with pPIC9 (data not shown) and 1440 base pairs for the clones transformed with pHIL-S1 (lane 5 and 9 in fig. 5-7).

38 His^+ transformants were analysed by PCR (Table 5-1). 24 of these contained the PGE sequence, 15 with an insertion and 9 as the result of a replacement. The fact that several His^+ transformants gave only an amplification of the *AOX1* gene can be explained by a recombination event in which the *HIS4** mutation in the *Pichia* genome is replaced by the corresponding *HIS4* region of the vector, without integration of the PGE insert.

Table 5-1 His$^+$ transformants of Pichia analysed by PCR

pPIC 9 transformants	His$^+$ Mut^S	His$^+$ Mut$^+$	pHIL-S1 transformants	His$^+$ Mut^S	His$^+$ Mut$^+$
8/1	-	-	30/1	-	+
8/2	+	-	30/2	-	-
8/3	-	+	30/3	-	+
8/4	+	-	30/4	-	-
8/5	-	+	30/5	-	+
8/6	-	+	30/6	-	+
8/7	-	-	30/7	-	+
8/8	-	-	30/8	+	-
8/9	-	-	30/9	-	-
8/10	-	+	30/10	-	+
8/11	-	-	30/11	-	+
8/12	-	+	30/12	+	-
8/13	+	-	30/13	-	+
8/14	+	-	30/14	-	+
8/15	-	-	30/15	-	-
8/16	-	-	30/16	+	-
8/17	+	-	30/17	-	-
8/18	-	-	30/18	-	+
8/19	+	-	30/19	-	-

5.4.2 Expression and detection of recombinant PGE

A few transformed *Pichia* strains of both types (insertion and replacement) generated after transformation with recombinant pPIC9 or pHIL-S1 plasmids were used for expression experiments. Non-buffered culture media were used for

growing the transformants. This results in a gradual decrease of the pH of the medium, inactivating proteases which are active at neutral pH (Cregg et al., 1993). The lower pH has no influence on *Pichia* nor on PGE because both are resistant to acidic pH.

Culture media and cell lysates were analysed for the presence of PGE on SDS-PAGE. No protein bands could be detected in the concentrated media after two or four days of induction. In the cell lysates so many protein bands were present, that no difference in protein pattern between the control strains and the transformed *Pichia* clones could be made. Therefore another approach was tested. PGE expression was screened by RT-PCR. For the transformants 8/3, 8/14 and 8/19 it was possible to demonstrate the presence of PGE poly(A)$^+$RNA (Fig. 5-8). This analysis was limited to only a few transformants because the isolation of intact total RNA and poly(A)$^+$RNA is time-consuming and difficult. These three transformants, together with a few others, were used in subsequent expression experiments (Table 5-2).

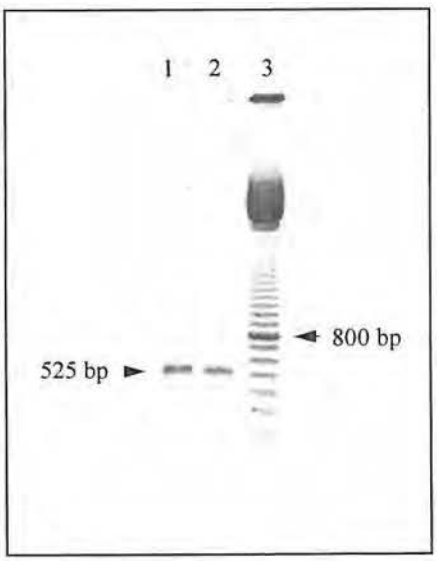

Fig. 5-8 RT-PCR analysis of transformant 8/14 (lane 1) and 8/19 (lane 2). Lane 3 represents the 100 bp ladder.

Table 5-2 Characteristics of the transformants and control strains used in the expression experiments

Transformants	Phenotype	Type of integration	Vector
8/14	$His^+ Mut^S$	replacement	pPIC9
8/19	$His^+ Mut^S$	replacement	pPIC9
8/3	$His^+ Mut^+$	insertion	pPIC9
30/1	$His^+ Mut^+$	insertion	pHIL-S1
30/8	$His^+ Mut^S$	replacement	pHIL-S1
Control strains			
HSA	$His^+ Mut^S$	replacement	-
β-gal	$His^+ Mut^+$	insertion	-

Immunoblotting was used to screen for the presence of the PGE protein. The first experiments with pAbs and mAb 3C9C7 showed a high background reaction. This made interpretation of the results difficult, because no specific reaction could be detected. In order to decrease the non-specific reactions the pAbs and mAb 3C9C7 against PGE and the AP-conjugated antibodies were first absorbed with the supernatant of a cell lysate of a control strain (1:2 overnight). The phenotype of the control strain (HSA: $His^+ Mut^S$ or β-Gal: $His^+ Mut^+$) always corresponded to the phenotype of the transformant used in the immunoblot.

In this way, a protein band with a molecular mass of about 65 kDa could be detected in the cell lysate as well as in the culture medium of the transformants 8/14 and 8/19 (Fig. 5-9 and 5-10). In the cell lysate this protein band was already present after 2 days of induction with methanol. In the medium this band was observed only after 5 days for transformant 8/19 and after 6 days for transformant 8/14.

The molecular mass of PGE calculated from its deduced amino acid sequence is 42.9 kDa (section 3.3.3). This implies that the recombinant PGE produced in *Pichia* is glycosylated for 34%, while in section 2.3.3 a carbohydrate content of 20% was found for PGE purified from calf tongue.

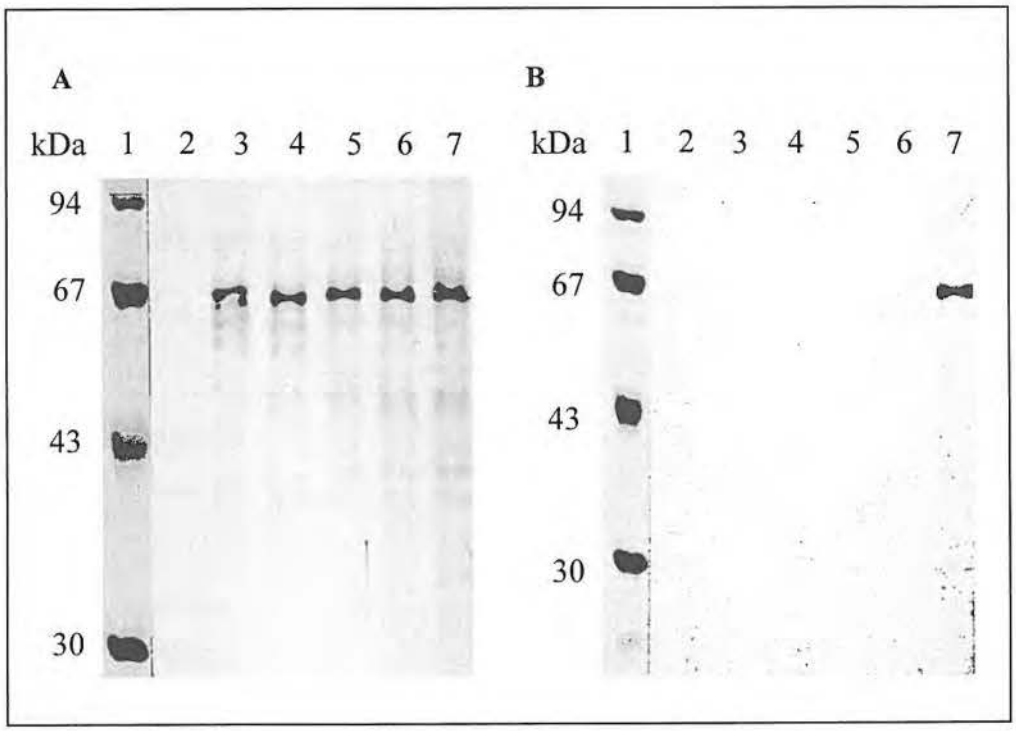

Fig. 5-9 Western blot analysis of the cell lysate (A) and the culture medium (B) of transformant 8/14.

Lane 1: molecular weight markers

Lane 2: control strain HSA after 6 days of methanol induction

Lane 3-7: transformant 8/14 after 2, 3, 4 ,5 and 6 days of methanol induction.

PGE was detected with mAb 3C9C7. For colour development anti-mouse-AP antibodies and BCIP/NBT were used.

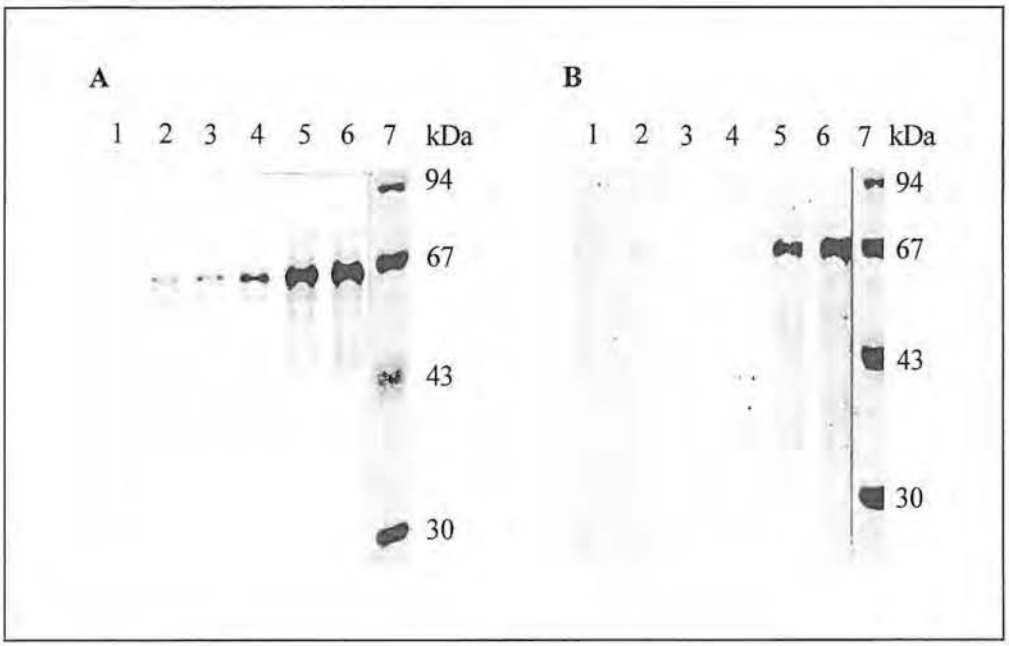

Fig. 5-10 Western blot analysis of the cell lysate (A) and the culture medium (B) of transformant 8/19.

Lane 1: control strain HSA after 6 days of methanol induction

Lane 2-6: transformant 8/19 after 2, 3, 4 ,5 and 6 days of methanol induction

Lane 7: molecular weight markers

PGE was detected with mAb 3C9C7. Visualisation was performed with anti-mouse-AP antibodies in combination with BCIP/NBT as a substrate.

5.4.3 PGE activity

PGE activity for transformants HSA, 8/14 and 8/19 was determined with PNPB and with tributyrin after 5 days of methanol induction. No activity was detected with a tributyrin emulsion, neither in the cell lysate nor in the culture medium. With PNPB, the cell lysate (10 µl) of transformants 8/14 and 8/19 showed a higher specific activity than the HSA clone

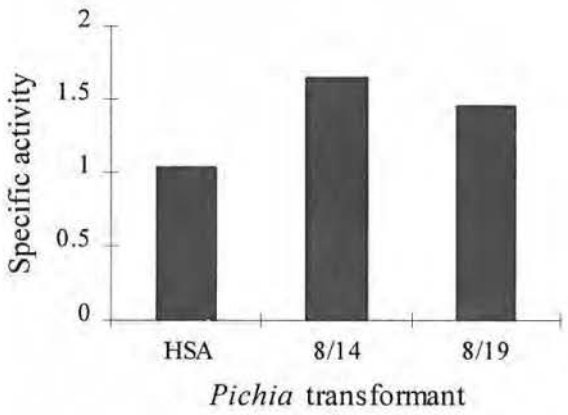

Fig. 5-11 Specific activities (OD_{405}/mg protein) of HSA, 8/14 and 8/19.

Recombinant PGE could be detected only by immunoblotting, indicating a very low expression level. This could explain why no reasonable activity was detected. Obviuosly a higher level of expression is required to investigate the activity of the recombinant PGE.

It has been reported that multi-copy integration of the expression cassette greatly improves the expression level (Clare et al., 1991). To select for multi-copy integrants a *Pichia* vector containing the kanamycin resistance gene will be used. This system allows the selection of multi-copy integrants by growing the transformants on medium containing increasing geniticin concentrations.

5.5 Summary

Two *Pichia* transformants generated with pPIC9 (8/14 and 8/19) produced PGE as a 65 kDa protein. This protein band was detected immediately after methanol induction in the cell lysate while it took about 6 days after induction to appear in the culture medium. Only the cell lysates of these two transformants showed a clear additional esterase activity as compared with the control strain.

6. Addendum

Expression of the pregastric esterase cDNA in insect cells: preliminary results

Autographa californica nuclear polyhedrosis virus (AcNPV) is a virus of the *Baculoviridae* family and is specific for insects. During AcNPV infection, two forms of viral progeny are produced: extracellular virus particles and occluded virus particles. The latter are embedded in proteinaceous viral inclusions, called polyhedra. The viral inclusions are an important part of the natural life cycle of the virus. The occluded virus particles are responsible for horizontal transmission among susceptible insects while the extracellular virus is responsible for secondary cell to cell infection.

Although the polyhedrin protein is one of the most abundant proteins in infected insect cells it is not essential for the infection or the replication of AcNPV under tissue culture conditions. For the expression of PGE in insect cells we chose the BaculoGold expression system of PharMingen (Smith et al., 1983). This system is based on baculovirus DNA that contains a lethal deletion in the polyhedrin gene and consequently does not produce viable virus particles (BaculoGold). Insect cells are co-transfected with the BaculoGold DNA and the transfer plasmid containing the PGE cDNA. By homologous recombination the lethal deletion of the virus DNA is rescued and gives rise to viable recombinant virus particles inside the transfected insect cells.

The PGE cDNA sequence, including its own signal peptide sequence was amplified by PCR and cloned in the transfer vector PVL1393. In this vector the expression is driven by the strong polyhedrin promoter and the expression cassette is flanked by viral sequences allowing homologous recombination at the polyhedrin gene of the BaculoGold virus DNA. The recombinant transfer plasmid was propagated in *E.coli* and the isolated plasmid DNA was used for co-transfection with BaculoGold DNA into *Spodoptera frugiperda* (Sf9) cells (Fig. 6-1).

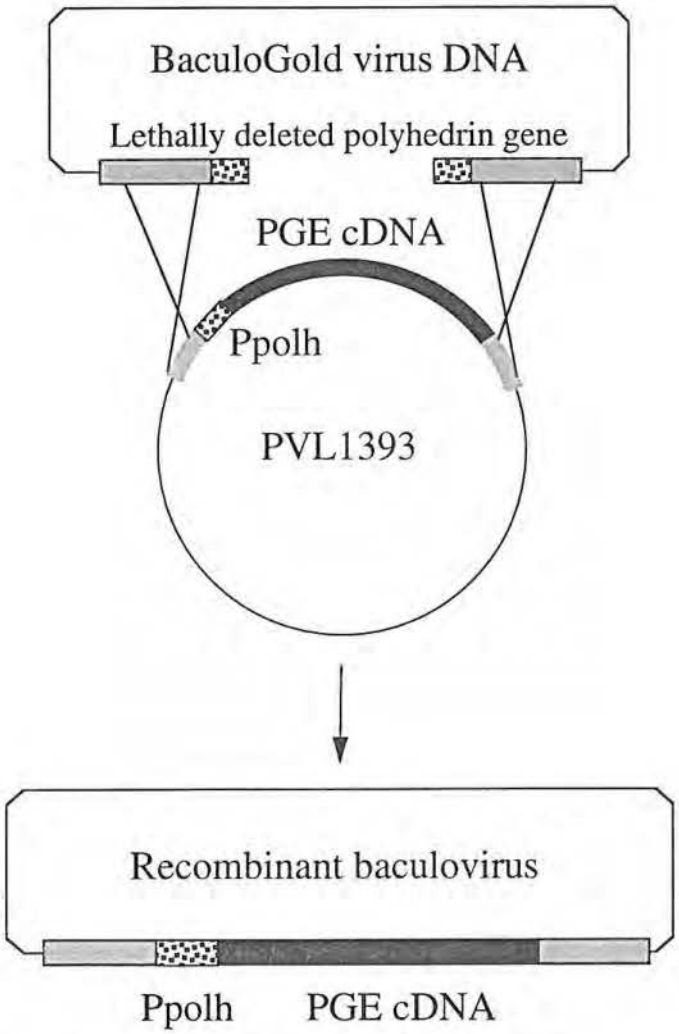

Fig. 6-1 Generation of recombinant baculoviruses

The medium of the co-transfected cell culture was collected and screened for the presence of recombinant virus. Insect cells infected with wild type baculovirus served as a control. Viral DNA was purified from the recombinant and wild type virus particles and subjected to PCR with primers complementary to the

polyhedrin locus. As can be seen in fig. 6-2 a 850 bp fragment is amplified with the wild type viral DNA and a 1850 bp fragment with the PGE baculovirus DNA.

To generate a PGE baculovirus stock solution Sf9 cells were infected with the co-transfection medium.

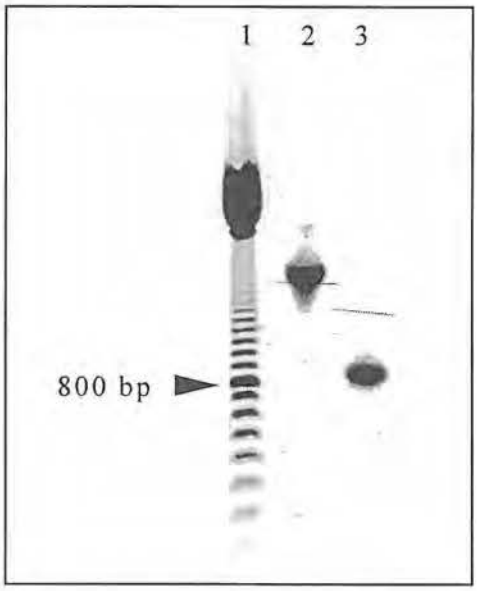

Fig. 6-2 PCR analysis of baculovirus DNA

Lane 1: 100 base pairs ladder marker

Lane 2: PGE baculovirus

Lane 3: Wild type baculovirus

Western blot analysis of the culture medium of PGE baculovirus infected Sf9 cells revealed a protein band of 45 kDa (baculoPGE). Endoglycosidase F treatment of this baculoPGE resulted in a band with a molecular mass of 40 kDa, indicating that baculoPGE is a glycoprotein with a carbohydrate content of 10% (Fig. 6-3).

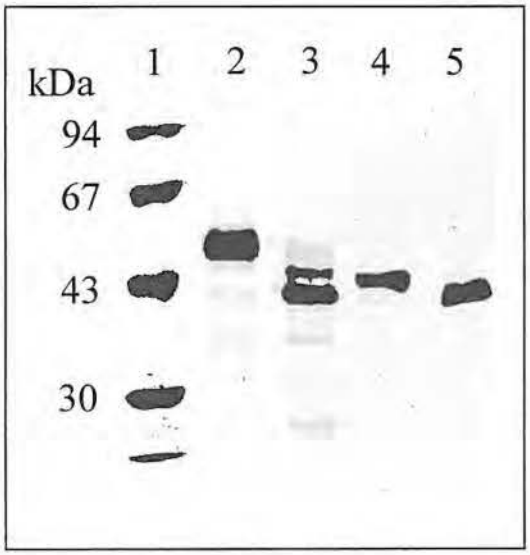

Fig. 6-3 Western blot analysis of the culture medium of PGE baculovirus infected Sf9 cells before and after endoglycosidase F treatment

Lane 1: native PGE

Lane 2: native PGE after endoglycosidase F treatment

Lane 3: BaculoPGE

Lane 4: BaculoPGE after endoglycosidase F treatment

In parallel with the immunoblot analysis, PGE activity was measured in the culture medium using tributyrin as a substrate. In the culture medium of Sf9 cells infected with PGE baculovirus a distinct lipase activity was measured, while in the culture medium of cells infected with the wild type virus no activity was detected (Fig. 6-4).

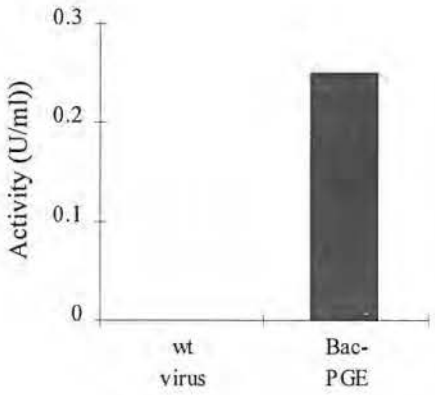

Fig. 6-4 Activity in the culture medium of Sf9 cells infected with wild type and PGE baculovirus, measured on a tributyrin emulsion.

Assuming that the specific activities of the native and the baculoPGE are identical, a yield of about 20 µg PGE per 10^7 Sf9 cells is obtained.

Addendum

7. General conclusions and future prospects

The lipase produced by serous glands in the papillae vallatae on the tongue and in the pharynx of the calf, better known as pregastric esterase (PGE), is a preduodenal lipase. It belongs to the acid lipase family. Acting mainly in the stomach, PGE is the major enzyme for the hydrolysis of dietary triacylglycerols in young animals. The enzyme is responsible for the hydrolysis of 65-70% of milk fat in the young ruminant (Gooden and Lascalles 1973).

PGE was purified about 13-fold by a combination of anion exchange chromatography, ammonium sulphate precipitation and gel filtration of extracts of calf pharyngeal tissue. In our purification procedure no delipidation step is required. All published methods for the purification of PGE and some other preduodenal lipases include this step for breaking up high molecular mass lipoprotein complexes of the enzyme. No such aggregation occurred in our procedure.

On SDS-PAGE the purified enzyme appears as a protein of 50 kDa. Deglycosylation of PGE results in a protein of 40 kDa, implying that carbohydrates represents about 20% of the total molecular mass.

With the aid of primers, constructed on the analogy between highly conserved regions in rat lingual lipase and human gastric lipase, the cDNA sequence encoding PGE was amplified by PCR from a calf tongue cDNA library. Sequencing of the amplified fragments resulted in the entire cDNA sequence for calf PGE (Timmermans et al., 1994). PGE contains a signal peptide of 19 amino acids and the mature enzyme is a 378 amino acid protein with a calculated molecular mass of 42,960 Da.

At the amino acid level PGE shows 70-75 % identity with rat lingual lipase and the gastric lipases of human, rabbit and dog. It has no identity with non-preduodenal lipases except for a Gly-Xaa-Ser-Xaa-Gly sequence which is conserved in all lipases. A central role for the serine residue in this pentapeptide sequence was predicted from X-ray diffraction analysis of some microbial and pancreatic lipases. We were able to prove the importance of a serine residue for

PGE activity by showing that dietyl p-nitrophenyl phosphate (E600) and tetrahydrolipstatin (THL) were potent inhibitors of PGE. In this respect the enzyme was not different from the other lipases.

However PGE is quite distinct from the gastric lipases with regard to the involvement of a free sulfhydryl group for its catalytic activity. Our experiments show that PGE possess only two cysteine residues, which are committed in a disulphide bridge. The enzyme has no free sulfhydryl group and consequently is not inhibited by the sulfhydryl reagents 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and 4,4'-dithiopyridine (4-PDS). These results are in contrast with those for gastric lipases. The gastric lipases contain three cysteine residues, of which only two are involved in the formation of a disulphide bridge. In accordance with the presence of a free sulfhydryl group, the gastric lipases are inhibited by the sulfhydryl reagents. PGE is the first lipase from the acid lipase family in which no essential free sulfhydryl group is present.

In the last part of this work we describe the results of experiments carried out with the intention of producing active PGE via recombinant DNA technology. In the first series of experiments PGE cDNA was expressed in *E. coli* as a fusion protein with glutathione-S-transferase. For this purpose we used the pGEX-5X-2 vector with an IPTG inducible *tac* promoter. It appeared that the major part of the fusion protein was produced under the form of insoluble inclusion bodies. The minor portion of the fusion protein, present in the soluble cell fraction, was purified by affinity chromatography on glutathione Sepharose. It had a molecular mass of about 64 kDa as determined by SDS-PAGE. Cleaving off the GST-moiety with coagulation factor Xa resulted in a protein with a molecular mass of 42 kDa. Neither the fusion protein nor the Xa cleaved protein showed lipolytic activity. A possible explanation for this inactivity could be the absence of glycosylation of proteins in a prokaryotic expression system. Because PGE is a glycosylated enzyme the carbohydrate component could be important for its catalytic activity. Therefore we assumed that eukaryotic expression would be a better way of producing enzymatically active PGE. Eukaryotic expression was performed in the yeast *Pichia pastoris* and in the insect cells *Spodoptera frugiperda*. *Pichia pastoris* is capable of metabolising methanol as a sole carbon source. Alcohol oxidase

(AOX) is responsible for the first step in the methanol metabolism. AOX expression is tightly regulated and induced by methanol. The methanol inducible AOX promoter is used in *Pichia* vectors to drive the expression of heterologous proteins. The PGE cDNA was cloned in pPIC9 and pHIL-S1, both equipped with a yeast signal peptide sequence (pPIC9: α-factor secretion signal of *S. cerevisiae*; pHIL-S1: *Pichia* acid phos-phatase secretion signal). By homologous recombination the PGE cDNA expression cassette was incorporated into the *Pichia* genome. In *Pichia* transformants, the PGE cDNA coupled to the α-factor of *S. cerevisiae* was expressed as a 65 kDa protein. By immunoblotting the enzyme was shown to be present in the cell lysate as well as in the culture medium. Only in the cell lysate, however, a small amount of activity was detected.

For the expression of PGE in insect cells the baculovirus expression system was used (see addendum on page 107). The PGE cDNA, including its own signal peptide sequence was cloned in PVL1393. By homologous recombination between the recombinant plasmid and the lethally deleted baculovirus DNA, viable baculovirus containing the PGE expression cassette was generated. With this system PGE was produced as a glycoprotein of 45 kDa, showing a distinct lipase activity.

Optimising the expression conditions could probably result in a larger production of active recombinant PGE. This would certainly allow a more detailed study of the enzyme. Site-directed mutagenesis for instance would then offer a very useful approach to determine which amino acid residues are important in the catalytic mechanism of PGE. X-ray diffraction studies on enzyme crystals would also provide much needed additional information.

Production of the recombinant enzyme on a large scale is also of interest to the food and dairy industry. The crude pharyngeal homogenates that are now always used by these industries could be replaced by the recombinant enzyme. In the medical sector the enzyme could find applications in the treatment of inadequate fat digestion due to pancreatic insufficiency.

8. Samenvatting en perspectieven

Vetten, meer bepaald triacylglycerolen, vormen een belangrijk deel van de voeding. De triacylglycerolen kunnen slechts opgenomen worden via de darmwand nadat ze gehydrolyseerd zijn door lipasen. Lipasen zijn speciale esterasen die in staat zijn om wateronoplosbare substraten te hydrolyseren. Dit onderscheidt hen van andere esterasen die alleen actief zijn op wateroplosbare substraten.

Tot voor kort werd de vetvertering bijna volledig toegeschreven aan de activiteit van het pancreaslipase, maar recent werd aangetoond dat preduodenale lipasen een belangrijk aandeel hebben in de vetvertering. De preduodenale lipasen zijn werkzaam in de maag en naargelang hun secretieplaats worden ze linguale, pharyngeale of gastrische lipasen genoemd. Het pharyngeaal lipase van het kalf, gekend onder de naam van pregastrisch esterase (PGE) is wel degelijk een lipase maar het is tegelijkertijd ook een esterase. PGE is een belangrijk lipolytisch enzym in het jonge dier. Het enzym is verantwoordelijk voor 65-70% van de melkvethydrolyse (Gooden en Lascalles, 1973).

In een eerste fase werd het PGE van het kalf gezuiverd uit pharynxextracten en gekarakteriseerd. De beste opzuivering werd bekomen door een combinatie van anionen-uitwisselingschromatografie, ammoniumsulfaatprecipitatie en gelfiltratie. Deze methode geeft een 13-voudige aanrijking in specifieke activiteit en een opbrengst van 14%. Het gezuiverde enzym is een eiwit van 50 kDa met een pH optimum van 5.5. Deglycosylatie resulteerde in een molecuulmassa van 40 kDa hetgeen impliceert dat suikermoleculen ongeveer 20% van de totale molecuulmassa vertegenwoordigen. Het grote voordeel van deze zuiverings-procedure t.o.v. van reeds eerder beschreven methodes (Bernbäck et al., 1985; Sweet et al., 1984) is dat er geen vetextractie nodig is om de vorming van aggregaten met een hoge molecuulmassa te vermijden.

Polyclonale en monoclonale antilichamen gemaakt tegen gezuiverd PGE kruisreageerden met het humaan gastrisch lipase en met het gastrisch lipase van

het konijn. Bovendien gaven antilichamen gemaakt tegen humaan gastrisch lipase ook een duidelijke reactie met PGE.

In tweede instantie werd het cDNA voor PGE gecloneerd en gesequeneerd. Uitgaande van poly(A)$^+$RNA, geïsoleerd uit tongweefsel, werd een cDNA bank bereid. Deze cDNA bank werd gescreend voor de aanwezigheid van PGE sequenties met de PCR techniek. De hiervoor gebruikte primers waren afgeleid uit de sterk homologe sequenties van reeds gecloneerde preduodenale lipasen, met name rat linguaal en humaan gastrisch lipase. De PCR produkten werden na subclonering in pUC18 gesequeneerd. Dit resulteerde in de volledige cDNA sequentie voor PGE (Timmermans et al., 1994). PGE bevat een signaalpeptide van 19 aminozuren en het rijpe enzym bestaat uit 378 aminozuren. Dit geeft een berekende molecuulmassa van 42.96 kDa wat goed overeenstemt met de molecuulmassa van het gedeglycosyleerde gezuiverde PGE, zoals door ons bepaald (40 kDa).

De PGE nucleotidensequentie en de afgeleide aminozuursequentie werden vergeleken met die van andere lipasen. Het PGE cDNA vertoont 70-75% homologie op aminozuurniveau met de andere preduodenale lipasen. Met de niet-preduodenale lipasen werd er geen homologie gevonden, behalve voor een geconserveerde Gly-Xaa-Ser-Xaa-Gly pentapeptide-sequentie, aanwezig is in alle lipasen die tot nog toe gesequeneerd zijn.

Aan de hand van X-stralen diffractie analyse van het lipase van de schimmel *Rhizomucor miehei* (Brady et al., 1990) en van humaan pancreaslipase (Winkler et al., 1990) werd aangetoond dat de serine-rest van dit pentapeptide deel uitmaakt van een Ser/His/Asp triade in de katalytische site. Tot nu toe is echter van geen enkel preduodenaal lipase de driedimensionele structuur opgehelderd.

PGE wordt geïnhibeerd door de serine-bindende reagentia diethyl-p-nitrofenylfosfaat (E600) en tetrahydrolipstatine (THL). Dit stemt overeen met de belangrijke rol van een serine voor de lipolytische activiteit van lipasen in het algemeen. Voor het gastrisch lipase van de mens en het konijn, geïnhibeerd door E600, werd aangetoond dat deze enzymen hun activiteit verloren tegenover wateroplosbare en geëmulgeerde substraten, maar dat ze nog steeds in staat waren om op een olie/water grensvlak te binden (Moreau et al., 1991). Dit laat

vermoeden dat de essentiële serine-rest betrokken is bij de katalytische werking en niet bij de substraatbinding.

In tegenstelling tot de gastrische lipasen wordt PGE niet geïnhibeerd door thiol-reagentia zoals 5,5'-dithiobis(2-nitrobenzoëzuur) (DTNB) en 4,4'-dithiopyridine (4-PDS). De gastrische lipasen bevatten drie geconserveerde cysteïnes terwijl PGE er slechts twee bezit. In de gastrische lipasen zijn twee cysteïnes betrokken in de vorming van een disulfide-brug. Het blokkeren van de overblijvende cysteïne met sulfhydryl-reagentia inactiveert de gastrische lipasen (Gargouri et al., 1988; Moreau et al., 1988; Carrière et al., 1989). De thiol-gemodifieerde gastrische lipasen kunnen nog steeds het substraat binden, maar ze zijn niet meer in staat het te hydrolyseren (Gargouri et al., 1989). Dit wijst erop dat de vrije sulfhydryl-groep van de gastrische lipasen belangrijk is in het katalytisch gebeuren. We hebben aangtoond dat de twee cysteïne-resten van PGE betrokken zijn bij de vorming van een disulfide-brug. Bijgevolg heeft PGE geen vrije cysteïne die kan tussenkomen in de lipolytische activiteit (Timmermans et al., in press).

In een volgende fase werd beoogd om het PGE cDNA functioneel en in voldoende hoeveelheden tot expressie te brengen. In eerste instantie werd het PGE cDNA tot expressie gebracht in *E. coli* als fusie-eiwit met gluthation-S-transferase. Hiervoor werd de pGEX-5X-2 vector gebruikt die voorzien is van een *tac* promotor waardoor de aanmaak van het fusie-eiwit geïnduceerd kan worden door IPTG. Het grootste gedeelte van het fusie-eiwit was aanwezig onder de vorm van inclusielichamen. Uit de oplosbare celfractie kon het GST-PGE fusie-eiwit gezuiverd worden door glutathion Sepharose affiniteitschromatografie. Het fusie-eiwit had een molecuulmassa van 64 kDa. Het verwijderen van het GST door te knippen met stollingsfaktor Xa resulteerde in een eiwit van 42 kDa. Noch het fusie-eiwit noch het geknipte eiwit vertoonden enige enzymactiviteit.

Daar PGE een glycoproteïne is, leek ons de expressie in een eukaryoot organisme meer aangewezen om actief enzym te produceren. Eukaryote expressie werd uitgevoerd in de gist *Pichia pastoris* en in de insectencellen *Spodotera frugiperda.*

Pichia pastoris bezit de meeste modificatie-, verwerkings- en transportmechanismen voor proteïnen die ook aanwezig zijn in hogere eukaryote cellen. Alhoewel deze mechanismen niet volledig identiek zijn aan die van zoogdiercellen zouden ze toch voldoende gelijkaardig kunnen zijn om de productie van enzymatisch actief PGE mogelijk te maken. *Pichia* is een methylotrofe gist die methanol als enige koolstofbron kan gebruiken. De eerste stap van de methanolafbraak gebeurt door het alcoholoxidase (AOX). De *AOX* promotor, die geïnduceerd wordt door de aanwezigheid van methanol wordt in de *Pichia* vectoren gebruikt om de expressie van heterologe eiwitten te sturen. Met het oog op secretie in het cultuurmedium werd het PGE cDNA gecloneerd in pPIC9 en pHIL-S1 vectoren waarin het voorafgegaan wordt door een gist signaalpeptide-sequentie (pPIC9: α-factor secretiesignaal van *S. cerevisiae*; pHIL-S1: zuur fosfatase secretiesignaal van *Pichia*). Door homologe recombinatie werd de PGE cDNA expressiecassette geïncorporeerd in het *Pichia* genoom. Met PCR werd de aanwezigheid van de PGE cDNA-sequentie in het gist genoom aangetoond. In transformanten, bekomen met pPIC9, werd PGE geproduceerd als een 65 kDa eiwit. Het eiwit werd zowel in het cellysaat als in het cultuurmedium aangetoond met Western blotting. In het cellysaat was het mogelijk een hogere PGE activiteit te meten dan in het cellysaat van de controles.

Voor de expressie in insectencellen werd het baculovirus systeem gebruikt (zie addenum pag. 107). Het PGE cDNA, met de eigen signaalpeptide sequentie werd gecloneerd in PVL1393. Homologe recombinatie tussen het recombinant plasmide en lethaal gedeleteerd baculovirus DNA resulteerde in recombinant baculovirus met de PGE expressiecassette. Met dit systeem werd PGE gesecreteerd in het cultuurmedium als een glycoproteïne van 45 kDa. Bovendien was het mogelijk een duidelijke lipase activiteit in het medium aan te tonen.

In een volgende fase zal het nodig zijn de expressiecondities te optimaliseren. Van zodra het mogelijk is om voldoende hoeveelheden actief PGE te produceren kan door middel van 'site-directed' mutagenese worden uitgemaakt welke aminozuren betrokken zijn bij het katalytisch mechanisme van PGE. Daarenboven kan actief recombinant PGE verschillende industriële toepassingen vinden, o.m. bij de bereiding van kazen. Ook zou recombinant PGE kunnen

gebruikt worden voor de behandeling van patiënten met een deficiënte vetvertering ten gevolge van pancreasinsufficiëntie.

9. References

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