PROTEIN ENGINEERING STUDIES OF OVINE BLG

SYNTHESISED IN YEAST.

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DECLARATION

I hereby declare that this thesis has been composed by myself, that the work it describes is my own, except where stated in the text, and that it has not been accepted in any previous application for a degree.

Gary J. Paterson

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Abbreviations

Miscellaneous

N-	amino
C-	carboxy
TBS	Tris-bufferred saline
GuHCl	guanidine hydrochloride
PEG	polyethylene glycol
oligo	oligonucleotide
glu	glucose
gal	galactose
PAGE	polyacrylamide gel electrophoresis
DTT	dithiothreitol
TE	10mM Tris-HCl pH 8, 1mM EDTA

Proteins

BLG	ß-lactoglobulin
A1AGP	α-1-acid glycoprotein
A1UG	α-1-microglobulin
A2UG	α-2-microglobulin
APHRO	aphrodisin
APOD	apolipoprotein D
BBP	bilin-binding protein
BG	Bowman's gland protein
C8	gamma subunit of complement component C8
CRC	crustacyanin

ESP	androgen-dependant epididymis secretory protein
HC	protein HC
INCYN	insecticyanin
MUP	major urinary protein
OBP	odorant-binding protein
PP14	placental protein 14
PUR	purpurin
RBP	serum retinol-binding protein
PGK	phosphoglycerate kinase
ADH	alcohol dehydrogenase
PCBs	polypeptide chain binding proteins

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Abstract

ß-lactoglobulin (BLG) is the major whey protein in ruminants and is found in the milk of a wide range of species. Extensive study of BLG over the years has led to the determination of the structure of the protein at 2.8Å and has revealed its ability to bind a variety of small hydrophobic molecules. However, the physiological function of BLG remains unknown despite its inclusion within the lipocalycin family on the grounds of genetic and tertiary structure comparisons as well as similarities in amino acid sequence.

A protein engineering approach was adopted to study the residues involved in subunit interactions and ligand binding as well as the importance of Cys119 and 121 in determining the stability of the tertiary structure.

The complete coding sequence for ovine BLG was obtained and a convenient site-directed mutagenesis system was set up. An expression system in the yeast *Saccharomyces cerevisiae* was developed in which ovine BLG was synthesised and secreted into the growth medium. Secretion was directed using the native BLG leader sequence. N-terminal sequencing of the secreted protein showed that the excision of the signal peptide occurred at the same site observed *in vivo*. Four mutant forms of the protein were generated. Cys119 and Cys121 were replaced with serine as separate mutants. Ile29, one of the residues at the subunit interface, was replaced with aspartate, and Lys70, located at the entrance to the hydrophobic pocket, was replaced with asparagine. All mutants, apart from 119, were secreted into the medium. Mutant 119 was detected in cellular extracts by Western analysis. This suggested that loss of the disulphide bridge between Cys106 and 119 sufficiently altered the structure of the protein to inhibit its passage through the secretory pathway. Although approximately 6-8mg of BLG were secreted per litre of yeast culture, further optimisation of the purification scheme was required to enhance yields. Consequently, only preliminary characterisation of the proteins was performed. Molecular weight determination using calibrated FPLC columns showed that all secreted BLGs appeared to be monomeric.

1.0 Introduction

ß-lactoglobulin (BLG) was first discovered in bovine milk more than 50 years ago, and it represents the major whey protein component in all the milks in which it is found. Milk is a substance rich in lipids, proteins, vitamins and minerals and provides essential nutritional requirements for newborn suckling mammals and marsupials. It is also a means of providing passive immunity in some species, eg artiodactyls, in which the newborn lack circulating antibodies. However, in many species where passive immunity is acquired through the placenta, eg rodents and primates, milk from these species still contains high concentrations of immunoglobulins which probably serve as an additional supplement. Milk can vary in content and concentrations of particular constituents depending upon the species, diet, health state of the animal and the time leading up to and after parturition.

The mechanism of milk secretion in mammals is well understood [1]. Milk is produced by epithelial cells that line the mammary alveoli and is stored in the alveolar lumina adjacent to these cells. There are five distinct pathways for the secretion of milk components.

1) Exocytosis of milk specific proteins and lactose from Golgi-derived vesicles.
2) Secretion of ions and water across the plasma membrane.
3) Secretion of fat via the milk fat globule.
4) Pinocytosis from the blood supply followed by exocytosis from the cell eg. immunoglobulins.

5) The paracellular pathway for plasma components and leucocytes.

Milk, therefore, contains ingredients that are either specifically synthesised in the mammary gland, derived from the blood supply, or accidently incorporated by the processes controlling the previous two. The biological function of many milk constituents has been determined, with one major exception. BLG, with a yield of between 2-4g per litre in bovine milk [2], is the major whey protein in a wide variety of animals. Although its properties have been extensively studied using a wide variety of techniques since its first isolation in 1934 [3], its biological function has yet to be determined.

1.1 Expression of BLG

BLG is thought to be expressed from a single copy gene in ruminants [4]. The ovine gene is composed of seven exons, the seventh exon being totally non coding [5]. The mature transcript from this gene, about 800 nucleotides long and representing up to 5% of the total mRNA in the mammary gland, produces a polypeptide 180 amino acids long [6]. This Pre-BLG polypeptide comprises the 162 amino acid mature polypeptide and the 18 amino acid signal peptide which directs the protein into the cell secretory pathway prior to being excised in the endoplasmic reticulum. Bovine BLGs are not normally subject to further post-translational modification as shown by the absence of glycosylation, even though there is a potential N-

glycosylation site, Asn-X-Thr, at Asn152. However, BLG from the Australian Droughtmaster breed of cattle is glycosylated at Asn-28 [7], the residues at this position from other variants being Asp.

The levels of mRNA coding for the six main milk proteins, αs1-, αs2-, ß-, k-casein, BLG, and α-lactalbumin, have been studied during the normal development of the mammary gland in sheep [6,8]. At mid-pregnancy, there are high levels of BLG mRNA which increase approximately 20 fold by day 20 of lactation. Casein mRNAs can barely be detected at mid-pregnancy and levels only increase slowly over the same time period, albeit in a coordinated fashion. Results have shown that although expression from all these genes can be induced by prolactin, and the levels modulated by glucocorticoids, eg. cortisol, BLG mRNA expression is regulated independantly from the casein genes.

1.2 Genetic variation and distribution

BLG from bovine milk was the first BLG to be characterised in detail. The puzzle concerning the heterogeneous nature of BLG crystals grown from supposedly pure protein was solved when BLG was found to consist of two variants that differed in electrophoretic mobility under nondenaturing conditions at pH 8.6 [9]. Since then, further research relying on starch gel electrophoresis, amino acid analysis and reactivity with anti-BLG antisera has not only revealed the presence of other bovine variants but has shown that BLG exists in the milk of a wide range of species, some of which also possess variants.

1.2.1 Ruminant variants

Ten different BLG variants have so far been isolated from bovine milk. Initially detected by their altered electrophoretic mobilities, the amino acid differences between nine of the variants have either been determined by sequencing or as a result of amino acid analysis of peptide digests. A comparison of the amino acid sequence variations of bovine BLGs are shown in figure 1.0. Individual cows can be homozygous for a particular variant or heterozygous. Since the variant BLG genes are expressed codominantly [4], heterozygous cows produce equal amounts of both variants in their milk. A and B variants are the most common. The C variant has only been isolated from Jersey cattle [15] and the D variant from two individuals of the Simmenthal breed [16]. E, F and G variants have all been isolated from Bali cattle (Banteng oxen) [12]. The E variant appears to have the same amino acid composition as the BLG from yak's milk [12] and another variant, W, recently isolated from a rare breed of Bavarian cattle, Murnau-Werdenfelder, has an electrophoretic mobility very close to that of the B variant but has been shown to differ by a single isoleucine to leucine substitution at position 56 [14,17]. Preliminary characterisation by amino acid analysis of another recently isolated bovine BLG, BLG H, has shown this particular variant to have the highest number of exchanges so far detected within the group [18], however, these results may not be reliable.

Figure 1.0 Variations in the amino acid sequences of bovine BLGs relative to BLG-B

	28	45	50	56	59	64	78	118	129/30	158	ref
BOV A	D	E	P	I	Q	**D**	I	**V**	D	E	[10]
BOV B	D	E	P	I	Q	G	I	A	D	E	[10]
BOV C	D	E	P	I	**H**	G	I	A	D	E	[2]
BOV D	D	**Q**	P	I	Q	G	I	A	D	**E**	[11]
BOV E	D	E	P	I	Q	G	I	A	D	**G**	[12]
BOV F	D	E	**S**	I	Q	G	I	A	**D<->Y**	**G**	[12]
BOV G	D	E	P	I	Q	G	**M**	A	D	**G**	[12]
BOV Dr	**N**	E	P	I	Q	**D**	I	**V**	D	E	[13]
BOV W	D	E	P	**L**	Q	G	I	A	D	E	[14]

Numbers show the position of the amino acid substitutions which are highlighted in bold text.

D<->Y shows that the substitution of aspartate by tyrosine occurs at either position 129 or 130.

Genetic polymorphism is exhibited among other ruminants. There are three ovine variants, A,B and C [19,6,20]. A and C variants have Tyr residues at position 20 where the B variant consists of His and A and B variants have Arg at position 148 where the C variant consists of a Gln. The B variant is identical to the only BLG so far isolated from mouflon [21]. BLGs isolated from buffalo and goat milk also appear to be homogeneous [22,23] whereas two variants have been isolated from pooled milk samples from red deer which have a more acid isoelectric point than bovine A and B variants [24].

1.2.2 Non-ruminant BLGs

Sedimentation equilibrium ultracentrifugation studies have shown that all ruminant BLGs exist as dimers at physiological pH and temperature, whereas those from non ruminants tend to be monomeric. Monomeric BLGs from horse, dog, dolphin, cat and pig have all been isolated and all exhibit genetic polymorphism. Feline milk may contain as many as twelve BLG variants [25]. These BLGs appear to be products from three separate gene loci each with its own allelic variations. An individual lactating female could quite possibly have as many as six different BLG variants in its milk. N-terminal sequence comparisons of three of the variants with bovine and equine BLGs have confirmed their membership within the family. The complete amino acid sequence for feline BLG II has recently been determined [26] which showed a 54% identity with bovine B and 68% with the recently revised horse II [26]. Horse has two variants both of which have been fully sequenced [27,28]. They share

limited amino acid identity, only 113 out of 162 amino acids, and horse II is four amino acids longer. However, the recently revised sequence for horse II showed that this protein was only 163 amino acids long and shared only 109 out of 162 residues with horse I. The presence of three feline gene loci and the two equine variants have probably arisen from gene duplication events. Analysis of the whey proteins from dolphin, dog and manatee have shown that dog and dolphin possess two variants and manatee one [29]. N-terminal regions of several of these variants have been sequenced and an incomplete primary structure of dolphin BLG A has been determined [30]. Donkey BLG [31] and dimeric kangeroo BLG [32] have been fully sequenced and appear homogeneous whereas pig has at least three variants, A,B and C [33,34], one of which has been fully sequenced [35]. Lack of cross reactivity against anti-bovine antiserum [35,36] suggests that pig BLG is structurally distinct from its bovine counterpart. However, this may reflect a general trend in which antisera raised against monomeric and dimeric forms fail to cross react, although feline BLG was detected by both horse and bovine antisera [25].

1.2.3 Animals lacking BLG

There is strong evidence that milk from rodents, llamas and humans do not contain BLG. An immunological search for BLG among artiodactyls, and other orders was carried out using anti-bovine BLG antiserum [37]. This study failed to detect BLG from primates and rodents among others. However, it also failed to detect BLG in pig whey, a species in which BLG is now known to exist. These

results therefore, cannot be used as conclusive evidence that these animals lack BLG in their milk owing to the fact they may possess monomeric BLGs which may not be detected by an anti-"dimeric" antiserum. The major whey protein in rats and mice is a cysteine rich, acidic protein with a molecular mass of 14kDa called whey acidic protein. Whey acidic protein has been fully sequenced and shows no sequence similarity to BLG [38]. The major whey proteins of guinea pig and llama have all been isolated and identified with other common milk proteins eg. serum albumin and lactalbumin. None correspond to BLG [39,40]. The evidence concerning the presence of a human BLG has been more contradictory. Initially, human milk samples were found to react with anti-bovine BLG antisera, following which, a protein with a molecular mass of 13.5kDa and a pI of approximately 5.1 was isolated and was thought to correspond to human BLG [41,42]. Further fractions with a molecular mass of 18kDa were found to react with an anti-bovine BLG antiserum, with the amino acid composition of one of these being in relatively good aggreement with that of pig BLG [43]. However, later studies regarding the purification of immunoreactive proteins revealed a cross reactivity with human lactoferrin [44,45]. It has also been shown by radioimmunoassays [46] and immunoelectrophoresis [45] that dietary bovine BLG ingested by human lactating females is accumulated in the mammary gland and secreted into milk. It is not clear at present whether this BLG is intact or present as fragments, or whether there is a specific uptake mechanism in the mammary gland or some general accumulation from the serum.

From the comparison of sequences in figure 1.1, it can be seen that the strongest similarity between mammal BLGs exists at the amino end of the protein. This may have arisen through evolutionary pressure to maintain sequences important in the putative ligand binding site or sequences involved in receptor recognition. Addition of the more evolutionary distant species, kangaroo, shows that it does not have the conserved N-terminal region. Figure 1.2 shows the percentage of amino acid identity among BLGs from different animals. It is interesting to note that the average amino acid identity between ruminant BLGs is approximately 98% (this figure does not take allelic variations into account) whereas that for non-ruminant mammals currently stands at around 61%. Apart from the gross variations in amino acid sequences, non-ruminant BLGs are also slightly heterogeneous in size. One can only question whether the different nutritional requirements of each species reflects alternative but similar ligands carried by BLG or whether BLGs from all species carry the same ligand and the protein sequences have diversified as a result of different selective pressures. The function of BLG is likely to be important otherwise the protein would not be maintained in such a wide variety of animals. Animals that lack BLG may either use another mechanism to provide the same function in milk or use an alternative route and/or mechanism eg. through the placenta. It is also possible that these animals have no need for this particular function of milk which might reflect a major difference in embryonic development. A human BLG analogue, placental protein 14, is expressed in the secretory endometrium and decidua during the first trimester of pregnancy. Although present in the serum,

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                                                                      60
ovine A     IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQK
mouflon     IIVTQTMKGLDIQKVAGTWHSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQK
goat        IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQK
buffalo     IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQK
bov B       LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQK
pig I       VEVTPIMTELDTQKVAGTWHTVAMAVSDVSLLDAKSSPLKAYVEGLKPTPEGDLEILLCK
donkey I    TNIPQTMQDLDLQEVAGKWHSVAMAASDISLLDSEEAPLRVYIEKLRPTPEDNLEIILRE
horse I     TNIPQTMQDLDLQEVAGKWHSVAMAASDISLLDSEEAPLRVYIEKLRPTPEDNLEIILRE
horse II    TDIPQTMQDLDLQEVAGRWHSVAMVASDISLLDSEEAPLRVYVEELRPTPEGNLEIILRE
feline II   ATLPPTMEDLDIRQVAGTWHSMAMAASDISLLDSETAPLRVYVQELRPTPRDNLEIILRK
              ....* .** ..*** *...**..**.****....**..*.. *.*** ..***.* .
kangaroo    VENIRSKNDLGVEKFVGSWYLREAAKT----MEFSIPLFDMDIKEVNLTPEGNLELVLLE
               . . .*.  . .* *.  . . .    ..   . .   .. .. ** ..**..* .

                                                                     117
ovine A     WENGECAQKKIIAEKTKIPAVFKIDALNENKVL--VLDTDYKKYLLFCMENS-AEPEQSL
mouflon     WENGECAQKKIIAEKTKIPAVFKIDALNENKVL--VLDTDYKKYLLFCMENS-AEPEQSL
goat        WENGECAQKKIIAEKTKIPAVFKIDALNENKVL--VLDTDYKKYLLFCMENS-AEPEQSL
buffalo     WENGECAQKKIIAEKTKIPAVFKIDALNENKVL--VLDTDYKKYLLFCMENS-AEPEQSL
bov B       WENGECAQKKIIAEKTKIPAVFKIDALNENKVL--VLDTDYKKYLLFCMENS-AEPEQSL
pig I       RENDKCAQEVLLAKKTDIPAVFKINALDENQLF--LLDTDYDSHLLLCMENA-SQ-EHSL
donkey I    GENKGCAEKKIFAEKTESPAEFKINYLDEDTVF--ALDSDYKNYLFLCMKNA-ATPGQSL
horse I     GENKGCAEKKIFAEKTQSPAQFKINALDEDTVF--YLDTDYKNYLFLCMKNA-ATPGQSL
horse II    GANHACVERNIVAQKTEDPAVFTVNYQGERKIS--VLDTDYAHYMFFCVGPPLPSAEHGM
feline II   RENHACIEGNIMAQRTEDPAVFMVDYQGEKKIS--VLDTDYTHYMFFCMEAPAPGTENGM
             .*  * .  . *..*. ** * ..  .*  .    **.**  ....*.  . .  ....
kangaroo    KTDR-CVEKKLLLKKTKKPTEFEIYISSESSYTFCVMETDYDSYFLFCLYN--ISDREKM
             ..  * .  .  ..*. *. * .  .*      .:.** ....*.        . .

                                                                     162
ovine A     ACQCLVRTPEVDNEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
mouflon     ACQCLVRTPEVDNEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
goat        ACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
buffalo     ACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHV
bov B       ACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI
pig I       VQQCLARTLEVDDQIREKFEDALKTLSVPMRIL--PAQLEEQCRV
donkey I    VCQYLARTQMVDEEIMEKFRRALQPLPGRVQIVPDLTRMAERCRI
horse I     VCQYLARTQMVDEEIMEKFRRALQPLPGRVQIVPDLTRMAERCRI
horse II    VCQYLARTQKVDEEVMEKFSRALQPLPGRVQIVQDPSGGQERCGF
feline II   MCQYLARTLKADNEVMEKFDRALQTLPVHIRIILDLTQGKEQCRV
              *.*.**  .*..  ***  **..*. ....    .   ..*
kangaroo    ACAHYVRRIE-ENKGMNEFKKILRTLAMPYTVIEVRTR--DMCHV
              .  .*    ...  ..*   *..*. .  .   .  . *
```

* :=> match across all sequences
. :=> conservative substitutions
- :=> gaps in the alignment arising from deletions or insertions not shared by all sequences

Figure 1.1 Alignment of BLG sequences using the clustal program.

	ov	mou	gt	buf	bov	pig	don	hol	ho2	fel	kan
ov	100	99.4	99.4	97.5	96.3	61.6	50.6	56.8	49.4	55.6	36.2
mou		100	98.8	96.9	95.7	62.3	51.2	57.4	50.0	56.2	35.5
gt			100	97.5	96.3	61.4	50,6	56.8	49.4	54.9	36.2
buf				100	98.8	63.5	50.6	56.8	49.4	54.9	34.9
bov					100	62.9	50.6	57.4	49.4	54.3	34.2
pig						100	44.0	52.2	44.0	47.2	28.8
don							100	66.0	94.5	66.3	25.7
hol								100	67.3	56.2	26.1
ho2									100	68.1	24.3
fel										100	24.2
kan											100

ov = ovine A
mou = mouflon
gt = goat
buf = buffalo
bov = bovine B
pig = pig I
don = donkey I
hol = horse I
ho2 = horse II
fel = feline II
kan = kangaroo

numbers = percentage of amino acid identity

average amino acid identity among ruminants =97.7%

average amnio acid identity among non-ruminant mammals =60.6%

Figure 1.2 Amino acid identity of BLGs from different animals.

nucleic acid hybridisation has shown that no other tissues synthesise the protein [47]. The cDNA encoding the complete 180 amino acid precursor has been fully sequenced [47] and the deduced mature polypeptide, 162 amino acids long, shares a 53% amino acid identity with horse BLG1, but unlike most BLGs, this protein is glycosylated [48]. Levels of placental protein 14 in the serum are highest between 6-12 weeks after fertilisation, whereas levels in the amniotic fluid are highest between 12-20 weeks [48]. This may suggest the presence of a specific transport pathway through the placenta to the developing embryo rather than allowing the protein to enter the general circulation of the mother. It is unlikely that the requirements for a developing human embryo during the first trimester supplied by placental protein 14 reflect those of a developed newborn calf so there can be no suggestion that placental protein 14 serves the same function as BLG. Like BLG, the function of placental protein 14 is unknown.

At the moment, the list of animals containing BLG in their milk is quite small but this is likely to increase with time as the search for BLG and BLG homologues gains momentum.

1.3 Isolation of BLG

Over the years BLG has undergone a battery of physico-chemical studies using a wide variety of techniques. BLG has been used as a general protein model to explain fundamental questions concerning the roles amino acids play in determining protein structure. As a result, large amounts of data have been compiled on the properties

of the protein which might give a clue to its physiological function. The extensive study of the protein has stemmed from the fact that BLG can be easily purified in large quantities from an almost limitless source.

Modern commercial preparations of BLG are based on a relatively simple technique developed by Aschaffenburg and Drewry [49]. Sodium sulphate is added to whole, or preferably skimmed, milk to precipitate fats, globulins and caseins among others, which are removed by filtration. The pH of the filtrate is reduced to 2 with concentrated acid causing rapid precipitation of protein constituents apart from BLG. The precipitate is removed by filtration and the pH of the supernatant is increased to around 6. BLG is precipitated by the additon of more sodium sulphate. Extensive dialysis of this resuspended precipitate against water eventually yields BLG crystals. This procedure was an improvement over the one developed by Palmer [3] where crystals formed from the "lactalbumin" fraction only after exhaustive dialysis at pH 5.2. Further improvements to the Aschaffenburg and Drewry method have been developed [50] where ammonium sulphate has been used as the precipitating salt. This avoids the careful temperature control required with sodium sulphate which has a tendancy to crystalise in whey solutions at lower temperatures. This method also reduces the effect of local pH excesses by using dilute acid and alkali and by increasing the pH of the acid precipitation step from 2 to 3.5. There are other isolation methods that do not rely on an initial acid precipitation step. Calcium chloride precipitation of caseins from skimmed bovine milk followed by dialysis, ion exchange

chromatography and gel filtration eventually yielded BLG crystals that diffracted better than those obtained following acid precipitation procedures [51]. Poorer diffraction from the earlier crystals possibly resulted from damage induced by the harshness of the acidic step. Another slight variation involving an acid precipitaion step at pH 4.6 to remove fats and caseins was used to isolate dolphin and manatee BLG [29]. Subsequent gel filtration followed by ion exchange chromatography provided BLG pure enough for amino acid sequencing.

1.4 Characterisation of BLG

Most studies have involved bovine BLG and all references to protein behaviour will concern BLG from this species unless otherwise specified.

1.4.1 Alterations in behaviour and molecular mass with pH

Variations in the molecular mass of BLG have been obtained using different procedures. Sedimentation equilibrium analysis has yielded values of 39kDa [52] and 35.6kDa [53], values obtained by osmotic pressure, 35.05kDa [54], and studies involving hydration of protein crystals have led to values of 35.8kDa in dry crystals [55]. However, it was shown that BLG undergoes rapid and reversible dissociation below pH 3.5, a reaction which is thought to involve both electrostatic and hydrophobic interactions. Light scattering measurements showed that both dissociation products had an approximate molecular mass of 18kDa [56]. This was supported by

a value of 17.1kDa obtained from surface films of BLG spread over concentrated ammonium sulphate solutions but this value increased to 34.3kDa in the presence of Cu^{2+} ions [57]. SDS-PAGE and amino acid analysis [58] showed the molecular masses to approximate 18.3kDa. Thus, the classsically defined protein with a molecular mass of 36kDa consists of two identical subunits of 18kDa which only dissociate into their monomer state at low pH.

An interesting behavioural phenomenon with one bovine variant was observed between pH 3.7 and 5.2 [59,60,61]. BLG A polymerises at low temperatures to form 144kDa octamers, or perhaps more accurately, tetramers of the dimeric form of BLG. Neither B nor C variants form appreciable aggregates over this pH range although polymerisation of A and B mixtures has been detected. The reaction is rapid and reversible but no appreciable amounts of aggregation intermediates were discovered. No larger aggregates were detected suggesting the formation of distinct structures rather than linear aggregates, the further polymerisation of these structures being possibly inhibited by steric hindrance. Carboxyl groups have been strongly implicated in this reaction. The pK of a carboxyl group lies within the optimal pH range of polymerisation and increased protonation might promote intermolecular hydrogen bonding. Modification of carboxyls with carbodiimide metho-p-toluene sulphonate at levels that induce little change in o.r.d. profiles with respect to the native protein greatly reduces the ability of BLG A to polymerise [62]. However, the inhibition is not total which suggests the involvement of other interactions. BLG A differs from other variants by having an Asp at position 64 which

might play an important role in this process. The Droughtmaster variant also has an Asp at this position but does not polymerise. This possibly arises from steric hindrance caused by the carbohydrate moiety attached to Asn 29.

BLG is primarily dimeric between pH 3.5 and 8. At higher pH values, the protein dissociates into monomers. The protein begins to denature as the pH increases and is accompanied by a large change in optical rotation [63]. The rate of change is slow at pH 8.25 but increases with increasing pH which coincides with the ionisation of amino groups. Infrared spectroscopy has shown that the primary stages of alkaline denaturation involve the unfolding of the α-helix and exposed ß-strands followed by the unfolding of core ß-strands to form a random coil structure. This rate of unfolding is enhanced at low temperatures [64]. The onset of rapid irreversible denaturation occurs above pH 9.5 and is thought to involve intra and interchain disulphide interchange. Studies showed that during prolonged incubation at pH 8.25, the free thiol content dropped to 7% of its original value and that aggregation was inhibited in the presence of thiol blocking agents [65]. Early sedimentation equilibrium studies showed a small decrease in sedimentation coefficient around pH 7.5 [52], but was not accompanied by a loss in molecular mass [66]. Titration curves showed the anomalous behaviour of two carboxyl groups, one per monomer, over the same pH range [67]. This unusually high pK for carboxyl ionisation inferred that the side chain was buried within the molecule below pH 7.5 and possibly involved in hydrogen bonding. Since BLG undergoes slow irreversible denaturation above

pH 8, this behaviour is possibly a result of refolding into an alternative motif more thermodynamically stable at this pH, rather than an unfolding of the molecule. The transition, commonly known as the Tanford transition, is totally reversible and does not appear to affect subunit interactions [66] (fig. 1.3).

1.4.2 Denaturation of BLG

Many denaturation studies lie outwith the normal environmental stresses of the protein and are not directly concerned with its physiological behaviour, although some lie within suitable ranges of pH and temperature. They supply information about the processes involved and whether denaturation by different means follows the same rules. Information derived from these studies can be important for the handling of the protein but many studies, especially extremes of temperature, are primarily useful to the food industry and will not be discussed here.

1.4.2.1 Effect of Temperature

Reversible conformational changes resulting from thermodenaturation are demonstrated by the changes in the environment of the two tryptophan residues, and occur up to and around 70°C prior to the formation of irreversibly denatured product [68]. Prolonged heating at 80°C followed by rapid cooling shows a similarity with alkaline denaturation in that there is a time dependant irreversible denaturation of the protein. This irreversible denaturation is characterised by the inability of one or both

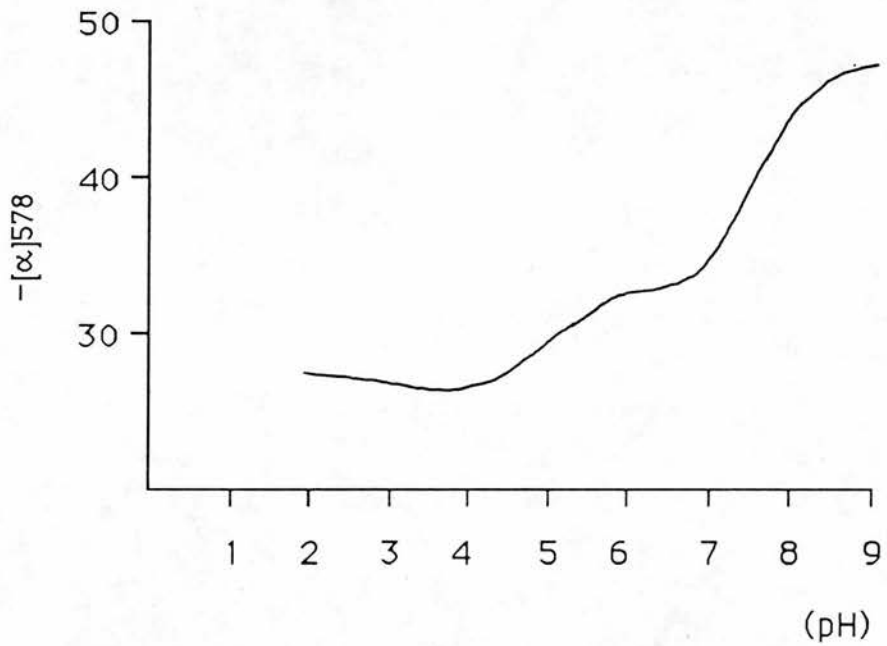

Figure 1.3 The Tanford transition. The diagram shows the dependance of the specific rotation at 578nm on pH of bovine BLG-B [178].

tryptophans to return to their hydrophobic environments. The first step involves the dissociation of the dimer [69]. It has also been shown that the denaturation temperature of BLG increases in the presence of sugars such as lactose and sorbitol [70]. Infrared spectroscopy [63] shows that reversible changes in conformation occur below 60°C and agrees with the similarities of alkaline denaturation observed above since some of the spectra obtained by both denaturation methods are superimposable. However, thermodenatured BLG still maintains a sizeable amount of secondary structure prior to aggregation which contrasts to the predominately random coil structures observed in alkaline denaturation.

The protein's thermodynamic behaviour was determined from equilibrium thermal unfolding curves derived from u.v. difference spectroscopy [71]. The observed properties were an indication of the strong acid stability of the protein. These investigations showed that the transition temperature from the folded to the unfolded state increased as the pH was lowered from 7.5 to 1.5, below which it decreased. These results, among others, led to the proposition of a possible mechanism for the acid stability of BLG [71]. As the pH is lowered from 7.5 to 5.2, the protein's isoelectric point, the loss of negative charge on the protein surface may facilitate closer packing within the protein's interior resulting in increased hydrophobic interactions and van der Waals attractions. This closer packing has been observed experimentally in which alterations in secondary structure induced by an increase in pH from 4 to 6 have led to an increase in sedimentation coefficient resulting from contraction of the protein [72]. As the

pH is lowered further, the gain in net positive charge does not result in the destabilisation of the protein. The stability of BLG increases down to pH 1.5 which may result from the loss of unfavourable localised electrostatic interactions as the carboxyls are titrated, which allows the formation of new and more stable hydrogen bonds upon protonation of the same groups.

Another important factor influencing thermodenaturation is the hydration of the protein. Hydration is essential for stabilising the spatial structure of proteins. Studies showed thermodenaturation is accompanied by the loss of ordered hydration shells around the protein with a concomitant decrease in the heat of denaturation [73].

1.4.2.2 Organic denaturants

BLG has been denatured by a variety of organic compounds such as urea, guanidine hydrochloride (GuHCl), alcohol and detergent. These studies have given rise to controversies regarding the kinetics of denaturation with individual denaturants as well as the conformation of the denatured protein.

Urea denaturation of BLG at pH 3 appears to follow first order kinetics which involve two major states, native and unfolded [74]. The first stage involves the complete dissociation of the BLG dimer at a urea concentration of 3M (BLG is already considerably monomeric at this pH). Since the major transition occurs between 4 and 6M, the native state was presumed monomeric. BLG is fully

denatured above 8M and the whole process is completely reversible. Other studies involving urea denaturation at pH 3.5 and 5.2 follow more complex kinetics [75]. Denaturation can be broken down into two parts: a rapid primary stage that is reversible and a slower secondary stage which shows an increasing loss of reversibliity with time accompanied by a gradual increase in viscosity. The kinetics of the refolding reaction increased in complexity with time but the reaction was more favourable at pH 3.5 than at 5.2. This increase in complexity was attributed to aggregation arising from disulphide interchange since denaturation assumed first order kinetics in the presence of N-ethylmaleimide, a thiol blocking agent. These studies led to a sequential rather than a parallel mechanism for unfolding. The increasing loss of solublilty in weak salt solutions of BLG incubated for progressively longer periods in urea before dilution has also been attributed to disulphide interchange [76]. Denaturation studies on mixed disulphide derivatives [77] also showed a deviation from a two state mechanism of unfolding whereas urea gradient electrophoresis showed a rapid two state transition at pH 7.2 [78]. Renaturing BLG gave rise to two bands, one corressponding to native BLG, the other to a slower refolding intermediate. This slower band was absent in BLG denatured at pH 2 where the amount of disulphide interchange should be negligible. These contrasting results may be balanced by considering that two-state denaturation transitions may not hold under all conditions [79].

The general interpretation for the fully denatured state of BLG denatured by urea and GuHCl, determined by o.r.d. studies, consists of an unfolded random coil structure [80,81,82]. This contrasts with the surprising evidence obtained from c.d. analysis in which there is a loss of α-helix but the amount of ß-sheet remains constant even at high concentrations of denaturant [83].

Alcohol has the ability to denature BLG much in the same way as urea and GuHCl but the reaction is not completely reversible [82]. This irreversibility is acompanied by an increase in viscosity with time when the ethanol concentration approaches 40% v/v suggesting aggregation has occurred. Unlike urea and GuHCl, ethanol has no special ability to disrupt hydrogen bonds. This leads to the formation of a structure with a high α-helix content upon denaturation [82,84]. The formation of this helical structure does not represent a smooth transition from ß-sheet, but occurs in two stages: unfolding of the native structure followed by refolding into a structure with a high helical content.

SDS and other anionic detergents can affect the conformation of BLG. At low SDS concentrations, 1 mole of BLG (dimer) binds two moles of SDS. C.d. spectra in the far ultra-violet region showed there did not appear to be any difference in conformation between native BLG and the SDS-bound form [85]. Modification of cysteines and surface tryptophans did not affect binding but it also showed that the native conformation of the protein was not a critical prerequisite for binding. These studies also showed that photooxidation of BLG greatly reduced the ability of BLG to bind

SDS. However, SDS-bound and native BLG photooxidised to the same extent at the same rate. The nature of the binding site has not yet been determined. At higher concentrations, 22 molecules of SDS or n-Octylbenzene-p-sulphonate were found to bind in an "all or nothing" fashion [86,87]. These studies suggest that although there are two major binding sites per dimer, BLG contains many weaker binding sites that are rapidly filled as the protein unfolds. Increasing the detergent concentration further leads to the formation of micellar structures arising from interactions between the detergent bound to the protein and the detergent in solution.

Variations in molecular mass with pH may not bear any relationship to BLGs function *in vivo*, but may just reflect the physical characteristics of pure protein in solution. However, changes in conformation with pH may reflect important stages of the protein's passage through the digestive system. At acidic pH values, the protein is stable and adopts a more compact conformation. This probably correlates with its ability to resist degradation by the gastric conditions in the stomach. Digestion studies in rats showed that BLG resisted degradation by gastric proteases, even after four hours, and with a stomach pH of 3.46 [88]. BLG's resistance to proteolytic cleavage by pepsin and gastric juices has also been demonstrated *in vitro* [89]. As BLG travels into the small intestine, the concomitant increase in pH induces a relaxation of the tertiary structure, and around pH 7.5, a change in conformation known as the Tanford transition occurs. With the proposal that BLG may function as a ligand carrier (see later), it

is interesting to speculate that having protected the ligand during its passage through the stomach, the protein now adopts a conformation that allows the presentation of the ligand to its receptor in the small intestine.

Much controversy surrounds the denaturation of BLG. It is not surprising that BLG may denature differently in the presence of different denaturants, ie. alterations in the kinetics of denaturation or the amount of remaining secondary structure. However, its is unlikely variations arise using a single denaturant unless the reaction conditions vary. It is not clear at present whether denaturation by urea is a two step process or whether denaturation is mediated by the formation of intermediates. Further study is required.

1.5 Structure and function of BLG

There is no direct evidence regarding the physiological function of BLG. The protein has no apparent enzyme activity, and purification procedures have never led to the isolation of a specific ligand. This may be a reflection of the harshness or unsuitability of the procedures used. Proposed functions have relied on circumstantial evidence centering on similarities with other proteins at three levels:

A) tertiary structure,

B) organisation of the encoding genes and

C) amino acid sequence.

1.5.1 Structure of BLG

Early studies by i.r. spectroscopy showed that BLG had a tightly folded secondary structure with a large amount of ß-sheet and possibly a small amount of α-helix [90]. C.d. studies showed the protein comprised approximately 10% α-helix with the rest of the structure being divided equally between ß-sheet, possibly anti-parallel in nature, and random coil [91]. This was in rough aggreement with values of 15% α-helix, 50% ß-sheet and 15-20% reverse turn obtained using secondary-structure-prediction algorithms [92]. The true nature of the protein's structure was only obtained with the onset of X-ray crystallography analysis. Data obtained at a resolution of 6Å showed that BLG was roughly spherical in shape with a diameter of 28Å, and that the subunits shared a dyad related symmetry. The first detailed structure was obtained at a resolution of 2.8Å [94,95]. It consists of a flattened barrel, closed at one end, made up from eight anti-parallel ß-strands, designated A-H, which is flanked at the C-terminal end by a three turn α-helix and another stretch of ß-strand, Strand I, (fig. 1.4). This structure consists of 7% α-helix and 51% ß-strand and varies only marginally from the data obtained from another crystal, grown under slightly different conditions, at a resolution of 2.5Å [51]. Both structures clearly show the presence of disulphide bridges between Cys66-160 and Cys106-119 with a free sulphydryl at Cys121. This latter fact is contested by peptide mapping experiments which show that Cys106 can form a disulphide bridge with both Cys119 and 121 in equimolar amounts [96]. The subunit interface occurs along strand I and is

Figure 1.4 Tertiary structure of bovine BLG at 2.8Å.

The ß-strands are represented by green ribbons and are labelled A-I. The α-helix is shown in white ribbon.

thought to involve hydrophobic interactions between Ile 29 and 147 and the stacking of imadizole rings of His146 [95]. Although the major structural elements have been clearly defined, N-terminal and C-terminal regions as well as several loops do not have clearly defined areas of electron density, presumably reflecting regions of high mobility [97].

1.5.2 Structurally related proteins

Several proteins show a very close structural resemblance to BLG. Insecticyanin is a protein that binds the chromophore biliverdin IX and is found in the hemolymph and integument of the tobacco hornworm. Its importance lies in the blue-green camouflage colouration it provides in conjunction with yellow pigmentation supplied by carotenoids. It is synthesised only in the larval stage [98]. Insecticyanin binds the chromophore so tightly that the protein must be denatured in order to release it [99]. The protein has been crystalised [100] and the structure determined to a resolution of 2.6Å [101]. The structure of bilin-binding protein, a protein isolated from white butterfly which also binds biliverdin, has also been determined [102,103]. Both structures exhibit the flattened barrel conformation made up from eight anti-parallel ß-strands flanked on the C-terminal side by a three turn α-helix. The barrel, like the one in BLG, is open to solvent at one end and closed at the other. Both proteins also possess two disulphide bridges. It is likely that these proteins serve as transporters for the chromophore since biliverdin is insoluble in aqueous environments at neutral pH [102].

The structure of serum retinol-binding protein has also been determined to a high resolution [104,105]. Retinol-binding protein, synthesised in hepatocytes, transports retinol from the liver to target tissues complexed to transthyretin, formerly called thyroxine-binding prealbumin. Having deposited retinol with the desired cell surface receptor, the retinol-binding protein-transthyretin complex dissociates whereupon retinol-binding protein is degraded in the kidneys. Retinol-binding protein has three disulphide bridges and the same barrel construction flanked by a four turn α-helix. Retinol-binding protein has only been crystalised with retinol bound to it, but unlike biliverdin which is bound to insecticyanin and bilin-binding protein in a compact configuration [101,103], retinol found in retinol-binding protein isolated from human plasma is the extended all-*trans* isomer [106].

1.5.3 Homologous proteins

Evidence regarding the membership of BLG within a particular family of proteins can be obtained by looking for similarities in amino acid sequence. Analysis of this kind has shown that BLG belongs to a family of proteins collectively known as the lipocalycins which are known to be able to bind a variety of small hydrophobic ligands.

Members of this family include three rodent proteins that may function as behavioural stimulants. Major urinary protein from mouse is synthesised in the liver and secreted in the urine [107]. Other members from the same protein family are also synthesised in

the mammary, lachrymal and submaxillary glands with each tissue expressing its own specific pattern of mRNAs [107]. Alpha-2-microglobulin is synthesised in the liver of male rats and excreted into the urine [110]. Aphrodisin has been isolated from the vaginal discharge of female hamsters and has an aphrodisiac effect on males [111]. The levels of these proteins are under multi-hormonal control. No active ligand has been detected with these proteins and yet aphrodisin maintains its behavioural activity under conditions condusive to the separation of a noncovalently linked ligand [111].

In close connection with these proteins are others that may be responsible for binding such elusive molecules. Rat odorant-binding protein, bovine pyrazine-binding protein and frog Bowman's gland protein are all exclusively associated with the nasal olfactory and respiratory mucosa and can bind a variety of hydrophobic ligands [112,113,114]. These proteins may help to solubilise and concentrate odorants in the mucus and allow receptor mediated transfer to cells in the olfactory cilia [111].

BLG has already been compared to proteins that carry chromophores eg. retinol-binding protein and insecticyanin. Other functionally related proteins include purpurin and crustacyanin. Chicken purpurin is synthesised in nerve cells within the retina and is known to bind retinol and heparin [115]. The protein is associated with the extracellular matrix and is important for cell adhesion and cell survival [115,116]. Crustacyanin isolated from lobster binds the blue pigment astaxanthin [117] and may provide the same

colour camouflage role observed in the two insect proteins. Apolipoprotein D is a major component of high density lipoprotein in human plasma. As well as being synthesised in the small intestine and liver, the major source of other apolipoproteins [118], mRNA has also been detected in the pancreas, kidney, brain and spleen among others [119]. Various functions have been attributed to the protein. Apolipoprotein D has been shown to bind lecithin:cholesterol acyltransferase which catalyses the synthesis of almost all the plasma cholesteryl esters [120]. However, molecular modelling studies have shown that apolipoprotein D may bind heme-related compounds similar to biliverdin [121] and other studies have shown that it can bind progesterone [122].

Other protein members may have roles that affect the immune response to pathogens. The polymorphic α-1-acid glycoproteins are abundant plasma proteins synthesised in the liver [123]. Plasma levels increase under a variety of pathogenic conditions eg. acute inflamation [124] and highly purified α-1-acid glycoprotein has an inhibitory effect on thrombin-induced platelet aggregation [125]. It also binds progesterone in a pH dependant manner at one particular site [126] and can form interactions with over 60 different drugs [127]. The terminal complement component C8 is involved in membrane attack resulting in cell lysis. The gamma subunit of this complex appears to belong to the family, however, its function has still to be ascertained since loss of this subunit does not affect cytolysis [128]. Another heterogeneous protein, protein HC, is found free in the cytoplasm and complexed to IgA and/or serum albumin [129] possibly by disulphide bridging [130].

Protein HC has been shown to inhibit the migration of neutrophils in endotoxin-activated serum [129]. α-1-microglobulin has an almost identical amino acid sequence to protein HC apart from an extension at the C-terminal tail. Both proteins contain a brown chromophore which is covalently linked to carbohydrate moeities [131].

The final member of the group (at the moment) is androgen-dependant epididymis secretory protein which accounts for the major proportion of proteins in the epidiymal luminal fluid [132]. Sperm gradually matures as it passes through the epididymis but requires constant androgenic support [133]. This maturation is accompanied by the binding of several epididymis secretory proteins, one of which has been fully sequenced [132], but the roles of these proteins have yet to be elucidated.

The amino acid sequence relationships between the proteins can be seen in figure 1.5. As well as the limited similarities between adjacent proteins, the alignment shows two regions in which all or the majority of proteins share the same amino acids. Gly17 and Trp19 (BLG numbering) are conserved in all proteins apart from rat α-1-acid glycoprotein where Gly17 is substituted by Asp (the human variant is shown in the diagram). In another region, Asp98 and Tyr99 are present in the majority. Spatially, these regions lie around the closed end of the calyx implying they play a role in maintaining a similar structure or are involved in ligand binding within the pocket [140].

```
        0                                                            49
A1AGP   Q......... C......... N......... ....GKW..I ..A..N.E..
APHRO   Q......... .......L.. .......... ....GKW.TI .IAADN.EK.
  OBP   A......... .....E.L.. .......... SE.NG.WRT. YI.ADN.EK.
  PBP   A......... .....E.... ........N. SEL.G.WRT. YIGS.N.EK.
 A2UG   EEA....... .....SST.. NL......DV .KLNG.W..I ..AS.KREK.
  MUP   EEA....... .....SST.. NF......NV .K.NG.W..I ..ASDKREK.
   BG   Q......... C..D...V.K .L........ .K..G.WY.I A.AS...CK.
  ESP   .......... .......V.K .F......DI SK..G.WY.I A.AS.....P
   HC   .......... .P.D.IQV.. NF.......I S...G.WY.L A.GS...C.P
   C8   Q......... .P...IQ.K. NF......D. ...AGTW... A.GS...C..
 PP14   .......... ......QTK. .L........ .K.AGTW.S. AMA..NIS..
  BLG   .......... ......QT.. .L......D. .K.AGTWYSL AMA...IS..
  PUR   Q......C.V ....SF.VK. NF......D. .RYAG.WYAL AK.KDPEGLF
  RBP   .......C.V ....SF.VKE NF......D. .R..GTWYA. AK.KDPEGLF
APO D   .......F.. GKCP...V.E NF......D. .KY.G.WYEI .K..P...EN
INCYN   .......F.. G.CP.VKPV. .F......D. S..AG.W.EI AK..P.E.EN
  BBP   .......... G.CP.VKPVD NF......D. S.Y.G.W.E. AK..PN..E.
CRC-A   D.IP...V.. GKCASV...D NF......D. .RYAG.WYQ. .....N.YQP
CRC-C   D.IP...V.. GKCASV.... .L........ ..YAG.WYQ. A....N.YQ.

        50                                                           99
A1AGP   .......... ..F......K .........Y ..R..QC... T.......EN
APHRO   ..IEEGG.LR .YF.HI.C.K .C.E..I.FY .....QC... TV.G.....N
  OBP   ....EGG.LR AYF.H..C.D EC.EL.I.F. VK.D..C... TVVG.K..ED
  PBP   ..I.ENG..R .YF..L...D E.G.V.F.F. VKRDG....V .V.A.K...D
 A2UG   ..IEENG.MR VF...I.VLE N..SL.FKF. .K..GEC.EL Y.VA.KT.ED
  MUP   ..IE.NGN.R .F...I.VLE N..SL..KF. ..RD.EC.EL ..VADKTEK.
   BG   .L..K..NM. A.V......L N.G.....T. ...E..C... .V..T..EK.
  ESP   .L..K...M. A.V..L.E.L ....L..TTT ...E..C... .V.AT..D..
   HC   .L.....RM. ..TL.L.E.. TE.....T.T RWR.G.C.E. ...YEKTD.D
   C8   .L.....R.. A.TL...P.. T......... R...G.C..V ..LY..TG..
 PP14   L.....APLR V....L.PT. PE..LEI.L. RWEN..C.EK KVL.EKTG.P
  BLG   LLD..SAPLR V....L.PT. PEG.LEI.L. .WENG.CA.K K..AEKT..P
  PUR   LQDNISA.Y. V.E.G.M.A. .KGRV.L... W..CADMA.. YT..D.T.PA
  RBP   LQDNI.A..S V.E.G.M.A. .KGRV.L... W..CADM... .T..D...PA
APO D   .G.CI.A.YS ..E.GK.... .......G.. ...EG....V ......T.PA
INCYN   .GKC..AEY. ....GK...V .N..V..G.. ...EGD.... .PDAK.T..G
  BBP   .GKC..AEY. ....GK...V .N..V..G.. ...EG....V ..D.K....G
CRC-A   ...CI...YE .......... K.T....... ...LK...KV YP..EF...H
CRC-C   ..KC...EY. ....GK.... K.T....... ...LK...K. YP...F..PH

        100                                                          149
A1AGP   GT........ .....G...F ..L.I..D.. .......... .......L..
APHRO   GTY.....T. .....G.N.F ..L.IT.D.I FF.N.N.D.. ..G..T....
  OBP   G.Y.....TD .....G.N.F .VLK.T.D.I FFHN.NVD.. ..G.......
  PBP   G.Y......D .....G.N.F .I........ V.HN.NVD.. K.G.TT.L..
 A2UG   GEY.....V. ....DG.NTF TILKTDYD.Y VM.HLIN... K.GETFQLM.
  MUP   GEY.....V. ....DG.NTF TI.KTDYDNF LM.HLIN... K.GETFQLM.
   BG   ........Y. .....G..E. .IVATDYD.F .M.F...K.. ...EV..T.K
  ESP   .KF....... .....G..E. .V.ATDY..Y AI...T.... ..G.V..TMK
   HC   GKFL....Y. ........E. .VV.T.YD.Y AI.FLT.K.. .......T.K
   C8   ..FL...... .....GA... .V.ETDY..F A.L.L..... .........K
 PP14   ..FK....I. ......ANE. ..LDTDYDNF L.LCL..... P.QS....CQ
  BLG   ..FK....I. .......N.. .VLDTDY..Y LL.C...S.. P.QS....CQ
  PUR   ...M.Y.G.A ..L..G.D.. WV.DTDYDNY AI.Y.CRSL. .DGSC.D.YS
  RBP   ...MKY.G.A ..L..G.D.. WIVDTDYD.Y A..YSCR.L. .DG.C.D.YS
APO D   ....KF.... .......... WILATDY.NY AL.YSC.... .....H.DFA
INCYN   K..M.F...G ........V. WVLATDYKNY AI.Y.C.Y.. PDKK.H...A
  BBP   K...K....G .......NV. .VL.TD.KNY .I.Y.C.Y.D .DKK.H.DF.
CRC-A   ..ID....FA AP........ .V.ETD...Y .C.YSCI..D ..YKS....A
CRC-C   ..ID....FA AP........ .ILE.D...Y AC.YSCI... ..Y.S....S
```

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      150                                                       199
A1AGP .VY..... T.E..... ......IP.. .........D .C.P......
APHRO .VAGK....L T.E..E.L.Q .A.E..IP.E N.......TD TC........
  OBP .VAGK...DL ....KQ.L.K LAEE..IP.E NI......TD TC........
  PBP .L..K..... ..E..EKF.K L.E..GI... N........E ..........
 A2UG .LYGR...DL SSDIKEKFAK LCE.HGI.R. NIIDL..KTD RCLQ......
  MUP .LYGR.P.DL SSDIKE.FAQ LCEEHGI.RE NIIDL...A. RCLQ......
   BG .L.GR....L .ED....F.. ..E.VG...E ..I....KAT .CVP......
  ESP .LYSR...D. ..E.L..FR. ....HG..E. ....L....T .CV.......
   HC .LYGR.P..L .E..LQ.FR. ..Q.VG.PED .IF.....GE .CVP..Q...
   C8 .LYAR.L... .D..L.GF.Q ..QE....ED .IF.....G. .C.A..Q...
 PP14 ...AR.L... .DE..QGF.. A...LP.H.. ......Q.EE .C........
  BLG ..L.R.P... .DE.LE.F.. AL..LPMH.. ..F.P.Q.EE .C........
  PUR .IFSR.P.GL PP..Q.IVRQ .QEE.CM..Q ..F.P....G .C........
  RBP .VFSR.PNGL PPE.Q.IVRQ .QEELC...Q .........G .C........
APO D WIL.R.PN.L PPET.....N .L...S..ID ....K....D .....C....
INCYN WI.S.S.V.L .G.TKE.V.N .L...S..ID ....K....D FSEAAC..S.
  BBP WVLSRS.V.L .GE.K..V.N .L...S.V.D ....K...SD FSEAAC....
CRC-A FVFSR.P... .....Q.... .VEK..AVF. .....V..S. FV.......C
CRC-C FIFSRS.... .........Q .V.K..A.F. .....V.... FV......SC

      200      209
A1AGP ..........
APHRO .........E
  OBP ..........
  PBP .........E
 A2UG .........R.
  MUP .........RE
   BG ..........
  ESP .........R.
   HC ..........
   C8 .........R.
 PP14 .........R.
  BLG ..........
  Pur ..........
  RBP .........L.
APO D .........L.
INCYN .Y......R.
  BBP ..........
CRC-A .Y........
CRC-C .Y........
```

. = gaps in the clustal alignment and/or amino acid residues that are not conserved.

Figure 1.5 Regions of amino acid sequence identity within the lipocalycin family. The proteins were aligned using the Clustal program and the diagram shows the amino acids shared between adjacent proteins as well as those that are highly conserved. Residues that were not conserved or shared by adjacent proteins (the evolutionarily most closely related proteins are adjacent to each other) were omitted from the diagram.

1.5.4 Gene structure

Comparisons at the level of gene organisation can be made to provide further evidence for membership within a common family. Comparisons of the structures of genes encoding a number of lipocalycin proteins have already been made [5], however, three have been singled out here because of their striking similarities. The gene structures of major urinary protein [108], BLG [5] and placental protein 14 [109] are all shown in figure 1.6. Although all the corresponding introns vary in size, all genes consist of seven exons with the seventh exon being totally noncoding. Corresponding exons are similar in size. Major urinary protein and BLG share two identically sized exons, and even more strikingly, BLG and placental protein 14 share four. All three genes produce mature polypeptides of 162 amino acids which are secreted from their cells of origin.

Comparisons of the gene structures for BLG and retinol-binding protein have shown that although retinol-binding protein comprises only six exons, analagous exons (BLG exon2 and retinol-binding protein exon3 and onwards) encode regions of protein that share the same structural elements with exon5 of retinol-binding protein being very similar to the fusion of exons5 and 6 of BLG [5].

The lipocalycin family includes proteins that have a low overall sequence similarity although there are obvious branching regions of shared amino acids linking them. Several members are known to share distinctive tertiary structures even with large variations in

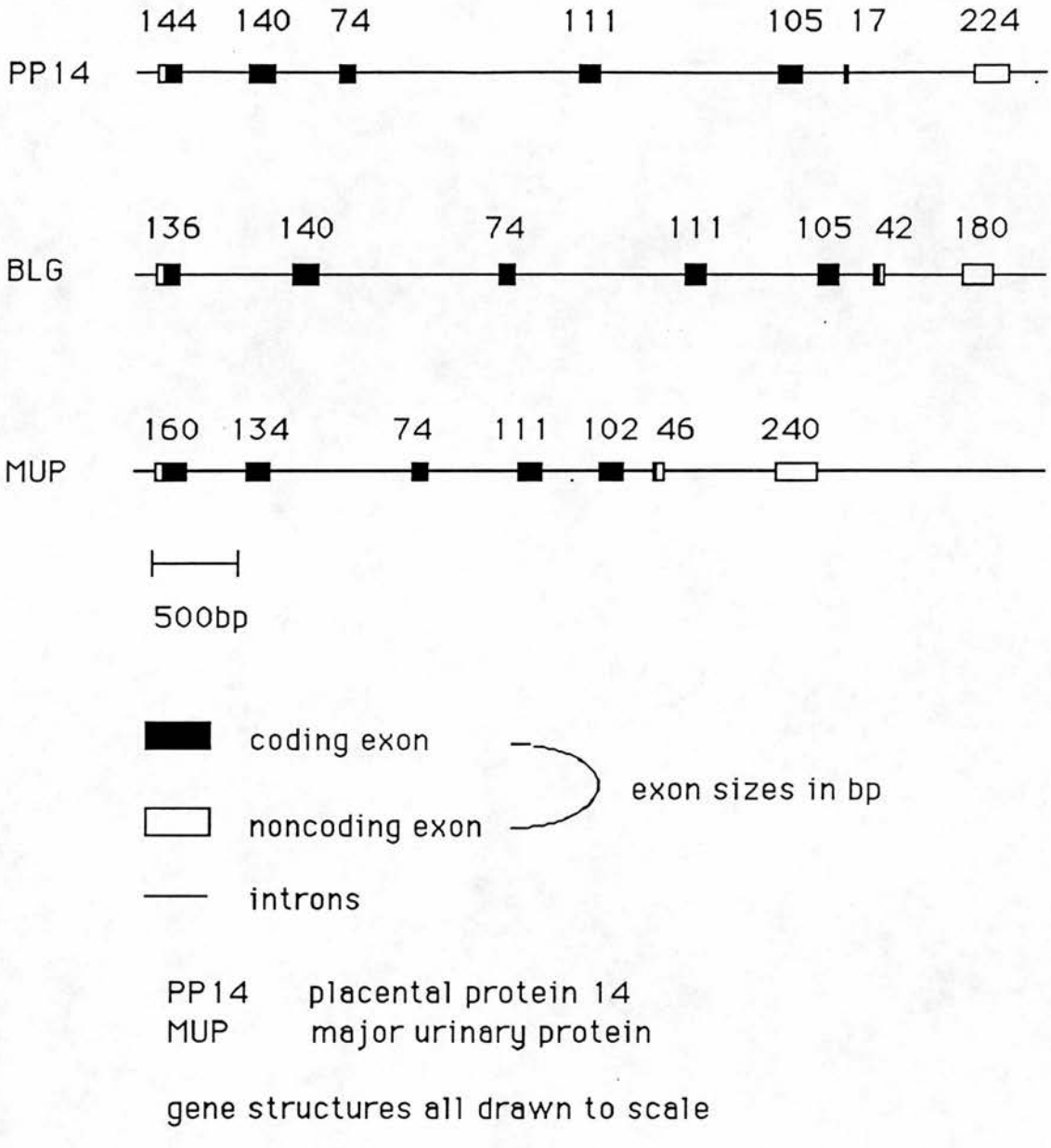


Figure 1.6 Alignment of gene structures of PP14, BLG, and MUP.

amino acid sequence. This may prove to be true for all members. The proteins are all small, around 20kDa, and comprise major components of plasma and/or other secretions. It is clear that BLG fits into this family using these criteria. Although there is a lack of evidence regarding the physiolgical role of many of these proteins, many have the ability to bind small hydrophobic molecules with varying degrees of specificity. These proteins may serve as general carriers for small hydrophobic molecules and it is possible that BLG has the same function.

1.5.5 Ligand binding

Since the finding that the structure of BLG closely resembles that of RBP [94], much attention has focussed on the possible role of BLG as a retinol carrier. The fluorescence lifetime and the fluorescence quantum yield of retinol bound to RBP is markedly greater than that of free retinol [141]. RBP also has the ability to protect retinol from oxidation by alcohol dehyrogenase. The binding of retinol to BLG produced the same effects but to a lesser degree in all three cases [137]. Spectroscopic characterisation of the retinol-BLG complex showed that two moles of retinol bound one mole of BLG dimer in a noncooperative manner [142]. Binding involved hydrophobic interactions and induced a rigidity in the retinol molecule. X-ray structural analysis has shown that retinol is completely enclosed within the calyx of RBP with the ionine ring lying deepest in the pocket and the isoprene tail stretching out to the surface [104]. Retinol has been modelled into a similar position with BLG but the isoprene tail is more accessible to

solvent [95] which might be a reflection on the protein's inability to protect retinol from oxidation. Interestingly, retinol-BLG complexes have been crystallised [51], and in this case the retinol is not bound within the calyx, but to a hydrophobic region on the protein exterior running parallel to the α-helix. The significance of this result is unclear at present.

Other evidence that BLG plays a role in retinol transport centres on the discovery of specific receptors for retinol-BLG complexes in the small intestine of neonate calves which do not appear to be present in adults [95]. This carrier role for BLG may correlate with the protein's ability to resist denaturation in the stomach. BLG can also enhance the uptake of retinol in the jejunum of young suckling rats [143] which is surprising since no BLG has ever been isolated from rat milk. This result is difficult to explain unless BLG in some way mimics the function of an indigenous rat protein [143]. However, since retinol is mainly associated with the fat globule [144] and no retinol-BLG complexes have ever been isolated other than those isolated *in vitro*, the binding of retinol is probably not specific but may reflect a general affinity for small hydrophobic molecules. Studies have shown that even retinol-binding protein can bind a variety of retinoids although only a few complexes are able to form interactions with transthyretin [106].

Other studies have proposed that BLG may play a role in lipid metabolism. Triglycerides are taken up from the blood to form milk lipids in the mammary gland [145]. Gentle BLG purification procedures have led to the detection of various free fatty acids

and triglycerides as well as trace amounts of cholesterol and cholesterol esters bound to the protein [146,147]. The free fatty acids and the fatty acids that make up the lipids consist mainly of myristic, palmitic and oleic acids with chain lengths of 14, 16 and 18 carbons respectively. These fatty acids account for the major proportion of lipids in bovine milk. Binding involves both electrostatic and hydrophobic interactions at one major site with the binding affinity increasing with increasing chain length to an optimum of 16 carbons [147,148]. Several weaker affinity sites are also present. However, serum albumin has a much higher affinity for lipids having as many as six high affinity sites as well as numerous weaker ones [148]. Albumin may serve as a general "mopping up" agent for free fatty acids since even small amounts of free fatty acids can be harmful to cells whereas BLG, with its weaker affinity, may facilitate their delivery to specific target cells.

It has also been reported that BLG can inhibit the hydrolysis of certain small phosphates by phosphoprotein phosphatase and may act to regulate phosphate metabolism in the mammary gland [149]. Spectroscopic studies showed that BLG could bind p-nitrophenol phosphate as well as other nitrophenyl compounds with a stoichiometry of one mole of ligand per mole of BLG monomer [150]. Studies involving other aromatic compounds showed similarities to the binding of free fatty acids and lipids, in that binding occurred at one high affinity site as well as several weaker ones [151].

The binding of small alkanes such as butane and pentane and others have also shown competition for a highly hydrophobic region near the surface [152], whereas a specificity for a structure formed by conjugated double bonds of the ß-ionone ring and its isoprenoid chain have been demonstrated during affinity studies on retinoid related flavour compounds [153].

It has been shown that BLG can bind a variety of small hydrophobic ligands. However, although some of these ligands are biologically important, there is no clear evidence that BLG functions as a specific carrier for any of them.

1.5.6 Milk protein interactions

BLG does not appear to form natural interactions with other milk proteins although lactoferrin can form soluble complexes *in vitro* in weak salt solutions [154]. It can form complexes with casein and lactalbumin resulting from heat induced coagulation [155,156] which creates problems for the food industry and will not be dealt with here.

1.6 Aims and strategy of the project

BLG has been the subject of extensive chemical modification studies in the past to answer questions regarding the particular roles of individual amino acids and how they might affect the structure and possibly the function of the protein. However, many modifications

and the conditions used to attain them may themselves have contributed to alterations in properties leading to misinterpretation, which on occasion has led to controversy.

It was proposed to develop an expression system using yeast to synthesise and secrete ovine BLG into the growth medium to facilitate purification of the protein. Specific amino acid residues would be altered by standard site-directed mutagenesis techniques and yields would hopefully be sufficiently high to allow full characterisation of all proteins.

Specific areas of interest lay with:

1) the amino acids involved in subunit interactions namely Ile29,
2) residues that might have a role in ligand binding namely Lys70

and 3) which cysteine residue is involved in forming the disulphide bridge with Cys106.

The long term aim of the project is to develop the protein as a drug carrier for hydrophobic or acid labile drugs. It may be possible to engineer the protein to bind a variety of drugs within the hydrophobic pocket and make use of the protein's acid stability to deliver the drug to the small intestine for absorbtion.

2.0 Materials and methods

2.1 Materials

2.1.1 Strains

E.coli: TG1 *K12, Δ(lac-pro), supE, thi-1, hsdDS/F'traD36, proA+B+, lacI^q, lacZ ΔM15.*

BW313 *HfrKL16 PO/45 [lysA(61-62)], dut-1, ung-1, thi-1, relA1*

S.cerevisiae: AH22 *leu2-3,2-12, his4-519, can1*

JRY188 *MATα, leu2-3,112, ura3-52, trp1, sir3-8, his4, rme*

DBY747 *leu2-3,112, his3A, ura3-52, trp1*

2.1.2 Vectors

pUC19BLG was supplied by Dr S. Harris who is currently at Glaxo Ltd, Greenford, Middlesex.

pK18, pUC1813, pUC1318 and pBM150 were all supplied by Dr A. Boyd from this department.

M13mp18 and mp19 were supplied by Bethseda Research Laboratories (U.K.) Ltd., Cambridge, U.K.

pMA91 was supplied by Dr S. Kingsman, Dept. of

Biochemistry, University of Oxford.

2.1.3 Growth media

Oxoid Ltd., Haverhill, Suffolk

Agar No.1, tryptone, yeast extract.

Difco Labs, Central Avenue, East Molesey, Surrey

Bactopeptone, purified agar, yeast nitrogen base without amino acids.

Sigma Chemical Company, Poole, Dorset

Ampicillin, kanamycin, Xgal, IPTG, all amino acids and nucleotides.

2.1.4 Enzymes

Northumbria Biologicals Ltd., South Nelson Ind. Est. Cramlington, Northumberland

Klenow fragment, T4 polynucleotide kinase and all restriction endonucleases.

Boeringher Mannheim, Bell Lane, Lewes, East Sussex

T4 DNA ligase and RNase A.

Amersham plc, Lincoln Place, Aylesbury, Buckinhamshire

T4 DNA polymerase, deoxyadenosine 5'(^{35}S) thiotriphosphate, triethylammonium salt, stabilised in

aqueous solution >400Ci/mmol.

Sigma

Lyticase.

2.1.5 Oligonucleotides

All oligonucleotides were synthesised by the OSWEL DNA service, Dept. of Chemistry, University of Edinburgh, West Mains Road, Edinburgh

2.1.6 Miscellaneous

Amersham plc.

Hyperfilm MP X-ray film and site-directed mutagenesis kit

Cambridge Bioscience, Newton House, Devonshire, Cambridge

Sequenase™ DNA sequencing kit.

Millipore (U.K.) Ltd., Peterborough Road, Harrow, Middlesex

Millex-GS 0.22um filter units.

Schleicher and Schuel, B-3354 Dassel, Germany

Biotrap apparatus, nitrocellulose.

Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hartfordshire.

Peroxidase labelled goat anti-rabbit antibodies.

Sigma

Polaroid 665 film, agarose and bovine BLG standard.

A & J Beveridge Ltd., 5 Bonnington Road Lane, Edinburgh

5,15 and 50ml Falcon tubes.

All other biochemicals were supplied by Sigma and B.D.H., Poole, Dorset, and were reagent grade or better.

2.2 Methods

2.2.1 Standard techniques

The standard experimental techniques used throughout much of this project are described in *Methods in Enzymology 1988, **152** ' Guide to Molecular Cloning Techniques*' and are listed below along with the page numbers.

Technique	page no.
Enzymic manipulation of DNA	91-144
Growth and storage of *E.coli*	145-151
Transformation of *E.coli*	151-154
Preparations of plasmids and phage	154-170
Sequencing of denatured plasmid DNA	556-562

2.2.2 Phosphorylation of oligonucleotides for mutagenesis

The oligonucleotides supplied by the OSWEL DNA service lacked the 5' terminal phospho group. This terminal phospho group is essential for the formation of a phosphodiester bridge during DNA synthesis of the mutant strand (see below).

40pmol oligonucleotide	1ul
10x kinase buffer	1ul
T4 polynucleotide kinase (3u)	1ul
distilled water	7ul

10x kinase buffer: 1M Tris-HCl pH 8, 100mM $MgCl_2$, 70mM DTT, 10mM ATP.

The reaction mixture was incubated at 37°C for 30 min then heated at 70°C for 10 min to heat inactivate the enzyme. The oligonucleotide could either be used directly for mutagenesis or stored at -20°C.

2.2.3 Site-directed mutagenesis

Two different methods for oligonucleotide-directed *in vitro* mutagenesis were employed in this study. Both methods required a single stranded DNA template, bacteriophage M13, which carried the gene to be mutated by a prior subcloning step. The life cycle of

bacteriophage M13 makes it extremely useful for these procedures in that its genome consists of a closed circular piece of DNA which replicates by means of a double stranded intermediate.

The Kunkel procedure

The template strand is grown in the BW313 strain of *E.coli* which carries the dut- and ung- mutations. The dut- mutation leads to a deficiency in dUTPase. Since dUTP cannot be converted to dUMP, there is an increase in the intracellular pool of dUTP which is then occasionally incorporated in place of dTTP during DNA synthesis. In ung+ strains, the incorporated dUTP would normally be removed by uracil N-glycosylase, however, this strain also carries the ung- mutation which results in the retention of incorporated uracil. Following the annealing of the mutant oligonucleotide and synthesis of the mutant strand *in vitro*, the double stranded phage is used to infect an ung+ strain, eg. TG1. Uracil is removed from the template strand which is then suseptible to exonucleases resulting in the destruction of the template strand. A new round of *in vivo* DNA replication should ensure that the desired mutation is now present on both strands.

The Eckstein procedure

This method was supplied in kit form by Amersham and all protocols were followed exactly. The protocol, briefly summarised, involves the anealling of the mutant oligonucleotide to the single stranded template like the procedure above. A phosphorothioate analogue of

dCTP is incorporated into the growing mutant strand during *in vitro* synthesis rendering it resistant to cutting by restriction enzymes such as NciI. The parental strand of the heteroduplex is then nicked by NciI making it susceptible to digestion by exonuclease III. A second round of *in vitro* DNA synthesis using the mutant strand as the template creates a homoduplex which contains the desired mutation on both strands.

2.2.4 DNA sequencing

The dideoxy method of sequencing supplied in kit form (Sequenase™ DNA sequencing kit available from Cambridge Bioscience) was used to sequence the wild type BLG gene and to verify the mutations generated in M13. For the purposes of this project, gene refers to the cDNA encoding the full length BLG polypeptide. The use of Sequenase™, a modified T7 DNA polymerase, allowed extended sequences of up to 500 bases to be read from a single primer.

Annealing reaction

DNA (1ug)	7ul
primer (4ng)	1ul
sequencing buffer (200mM Tris.HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl)	2ul

heat at 65°C for 2 min, slowly cool over a period of 30 min to 35°C.

Labelling reaction

To previous mixture add

dithiothreitol	1ul
labelling mix	2ul
[$-^{35}$S]dATP	0.5ul
Sequenase	2ul

Mix well and incubate at room temperature for 5-10 min.

Termination reaction

Add 2.5ul of the four different termination mixtures to four separate Eppendorf tubes.(Each termination mixture consists of 4 x 80um dNTPs and 8uM of a single ddNTP, 50mM NaCl)

Add 3.5ul of the above labelling reaction to each termination mix and incubate at 37°C for 5 min.

Terminate reactions with 4ul of stop solution (95% formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF).

Samples can be stored at -20°C or heated at 80°C prior to electrophoresis.

Plasmids rescued from yeast and prepared for sequencing by the method cited in the standard techniques section gave poor results. Double stranded DNA was prepared in the following way. Rescued plasmid was digested by SalI and EcoRI and the fragments separated by gel electrophoresis. A 3kb fragment containing the BLG gene was purified from the gel by electroelution using the Biotrap apparatus then precipitated using 0.4 vols of 5M ammonium acetate and 2 vols of isopropanol. Approximately 7ug of this fragment in 15ul TE (10mM Tris-HCl pH 8, 1mM EDTA) was denatured by adding 5ul of 1M sodium hydroxide, 1mM EDTA, and the mixture incubated at RT for 10

min. Instead of neutralising the mixture and ethanol precipitating the DNA, alkali was removed by spin dialysis. 20ul of sterile 200 micron glass beads in TE were added to a 500ul Eppendorf tube followed by 150ul of Sepharose-6CBL equilibrated in TE. A hole was punched at the bottom of the tube with a needle. The tube was inserted into a 1.5ml Eppendorf tube and spun at 1000rpm for 4 min in an MSE benchtop centrifuge to remove excess fluid. The DNA solution was added to the top of the column, spun as above and collected in a fresh 1.5ml Eppendorf tube. The eluted DNA was immediately sequenced using the Sequenase™ kit.

Sequencing gels were run on a BRL SO sequencing system then fixed, dried down on 3MM paper and autoradiographed at RT using Hyperfilm MP.

2.2.5 Growth of yeast

Two different kinds of media were used throughout these studies to grow yeast: a rich non selective medium that allows rapid growth and a synthetic selective medium which allows selection for cells with an autotrophic marker supplied by a transformed plasmid.

Rich non selective medium (YPD)

Yeast extract	1% (w/v)
Bactopeptone	2% (w/v)
Glucose	2% (w/v)

Glucose was replaced with 2% (w/v) galactose to induce expression from pBM150 derived plasmids.

Synthetic selective medium called yeast omission medium (YOM)

Yeast nitrogen base (without amino acids)	0.67% (w/v)
Glucose	2% (w/v)

This medium was supplemented with amino acid stock solutions.

100X omission stock:

	g/100ml		g/100ml
Adenine	0.1	Phenylalanine	0.25
Uracil	0.1	Glutamic acid	0.5
Arginine	0.1	Aspartic acid	0.5
Methionine	0.1	Valine	0.75
Histidine	0.1	Threonine	1.0
Tyrosine	0.12	Isoleucine	1.5
Lysine	0.12	Serine	1.75

Leucine stock solution: 0.5% (w/v) leucine

Tryptophan stock solution: 0.2% (w/v) tryptophan

Each stock solution was made up in distilled water, sterilised by autoclaving and stored at 4°C. Tryptophan is light sensitve and was covered with tin foil as an extra precaution. To produce the desired selective medium, 1ml of each of the stock solutions was added to YOM with a final volume of 100ml. Uracil and leucine were omitted from the medium as required when selecting for uracil and leucine autotrophs.

All of the above media were made up to the required volume with distilled water and sterilised by autoclaving. Glucose, galactose and glycerol were all autoclaved separately to avoid caramelisation.

2.2.6 Transformation of yeast

Transformation was performed by the lithium acetate procedure.

Solutions:

LA 0.1M lithium acetate in TE

PEG 50% PEG 4000, filter sterilised

A small overnight starter culture was grown overnight in YPD at 30°C. This was then subcultured into 50ml of fresh YPD to give an initial cell density of $1x10^6$/ml and grown at 30°C to a density of approximately $1x10^7$/ml ($3x10^6$ cells/ml = 0.1 at 600nm). The cells were harvested by spinning for 5 min at 2000rpm in an MSE benchtop

centrifuge, resuspended in 10ml TE, respun, and finally resuspended in 5ml LA. The cells were shaken at 30°C for 1 hr and then dispensed into 300ul aliquots in Eppendorf tubes. Up to 10ug of plasmid DNA was added in 10ul to the tubes followed by 700ul of PEG. The tubes were inverted gently to mix, placed in a 30°C water bath for 1 hr then heatshocked at 42°C for 5 min. At this stage PEG could either be removed from the cells by centrifuging gently and resuspending in TE, or 200ul of the cell/PEG solution could be plated out directly onto selective plates (whichever method was used, there did not seem to be any difference in transformation efficiency). The plates were then incubated at 30°C and colonies appeared within three to five days.

2.2.7 Plasmid rescue from yeast transformants

Colonies obtained from selective plates must be analysed to ensure they result from transformation of the desired plasmid and are not the result of contamination. The plasmid must be rescued from yeast and transformed back into *E.coli*. Following isolation from *E.coli*, the plasmid can be correctly identified by restriction mapping.

A 5ml culture of cells was grown overnight at 30°C in selective medium. The cells were harvested in a microcentrifuge and resuspended in 800ul BME buffer (0.9M sorbitol, 50mM sodium phosphate pH7.5, 2mM 2-mercaptoethanol). Lyticase (50u) were added and the cells incubated at 30°C for 20 min, or until spheroplasts had formed (add 5ul of 10% SDS to 5ul of cells, greater than 90%

lysis is a good indication of spheroplast formation). Spheroplasts were spun down at low speed in a microcentrifuge and resuspended in 100ul of 1M sorbitol. Lysis buffer (800ul) was added (100mM Tris-HCL, pH 9.7, 50mM EDTA, pH 8.5, 0.5% SDS) and the tubes were incubated at 70°C for 20 min. Potassium acetate (200ul of 5M) was added and the tubes were then incubated on ice for 45 min. The tubes were spun at high speed in a microcentrifuge for 1 min and the supernatant carefully removed, the pellet being discarded. Following the addition of 550ul of isopropanol, the tubes were left at room temperature for 5 min and then spun at high speed in a microcentrifuge. The pellet was washed in 80% ethanol, dried and resuspended in 20ul TE. 10ul was then used to transform *E.coli* in the usual manner.

2.2.8 Small scale preparation of crude yeast extracts

Cells were grown to late log phase and 1.5 ml were harvested in an Eppendorf tube. The cells were resuspended in buffer (100mM Tris-HCl, pH 7, 5% SDS, 20mM EDTA, 0.5% 2-mercaptoethanol) and glass beads (0.45mm diameter) were added to just below the meniscus. The tube was then vortexed for two to three minutes and then boiled in a water bath for 2 minutes. A small hole was then punched at the bottom of the tube with a needle. The Eppendorf tube was inserted into a 5ml Falcon tube and spun at 3000rpm for 5 min in an MSE benchtop centrifuge. The suspension was removed from the Falcon tube and respun in an Eppendorf tube to pellet cellular debris. The crude cell extract was either analysed directly by SDS-PAGE or stored at -20°C to limit proteolysis.

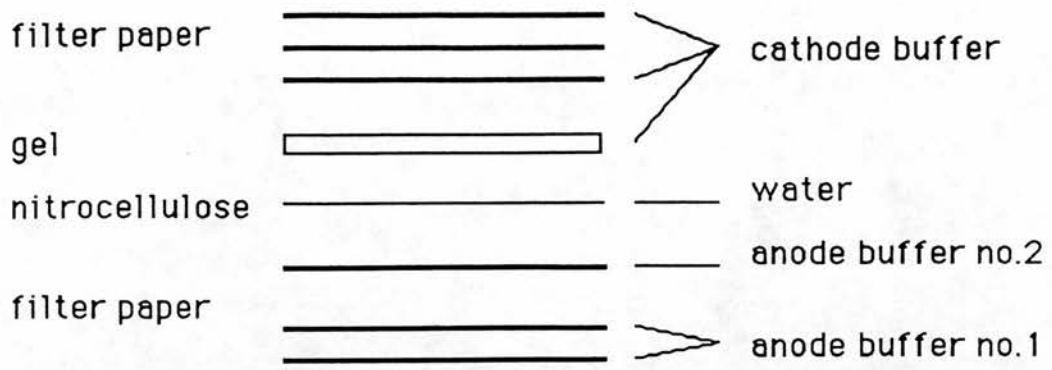
2.2.9 Denaturing gel electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Schagger and von Jagow [177]. Protein samples were prepared by mixing with an equal volume of boiling mix (10% (v/v) glycerol, 10% (v/v) stacking gel buffer, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.005% (w/v) Bromophenol Blue), boiling for 3 min and centrifuging for 10s at high speed in a microcentrifuge prior to loading.

Native PAGE was performed using the same system except that no SDS or 2-mercaptoethanol was used, the pH of all buffers was 8.6 and the samples were not boiled before loading.

2.2.10 Western blotting

Crude yeast cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose by a semi dry procedure using an LKB transfer system. A nitrocellulose membrane and six pieces of 3MM filter paper were cut to the exact size of the gel. Two pieces of filter paper were soaked in anode bufffer No.1, one piece in anode buffer No.2 and the other three in cathode buffer. The gel was equilibrated in cathode buffer for 5 min and the nitrocellulose membrane was gently immersed in water to avoid the formation of air bubbles. The filters and the gel were stacked in the order below.



Transfer was accomplished after 1 hr at a current determined by the formula:

Area of gel x 0.8mA.

Anode buffer	0.3M Tris, 20% methanol
No.1	0.1% SDS, pH 10.4
Anode buffer	25mM Tris, 20% methanol
No.2	0.15 SDS, pH 10.4
Cathode buffer	25mM Tris, 20% methanol
	40mM 6-amino-n-hexanoic acid
	pH 9.4

The membrane was washed in Tris buffered saline (TBS) and blocked for 1 hr in TBS + 0.5% Tween 20. Many of the standard blocking agents, ie. Marvel, BSA and gelatin, contain trace amounts of BLG and were unsuitable for immuno analysis. The membrane was incubated with the primary antiserum, rabbit anti-bovine BLG (kindly donated by Alan McAlpine), for 1 hr (or quite often overnight at 4°C) and then washed five times in blocking buffer.

The secondary antiserum (peroxidase conjugated anti-rabbit IgG) was added in blocking buffer at a dilution of 1/2000 and incubated at RT for 2 hr. The membrane was washed five times in blocking buffer and then developed.

Developer disolve 40mg 4-Chloro-1-napthol in 300ul methanol, add to 100ml 50mM Tris-HCl pH 7.5 and 100ul 30% hydrogen peroxide, filter.

This developer was later superceded by the highly sensitive Amersham ECL procedure. Using this procedure, the secondary antiserum was incubated for only 20 min at an increased dilution of 1/5000 and the percentage of Tween 20 was increased to 1% in the blocking solution.

2.2.11 H.p.l.c. and amino acid sequencing

Medium constituents plus the secreted BLG were concentrated using Amicon ultrafiltration cells and separated by reverse phase h.p.l.c. using an Applied Biosystems 130A Separation System. N-terminal sequence analysis was performed by Edman degradation using an Applied Biosystems 477A Protein Sequencer.

2.2.12 C.d. spectroscopy

Spectra were carried out on a Jasco J600 spectropolarimeter using 0.1cm path length quartz cells. Samples were scanned from 260-205nm at a temperature of 20°C. The c.d. analysis was performed by Sharon Kelly at the Department of Biological and Molecular Sciences, University of Stirling.

3.0 Results

3.1 Subcloning the BLG gene

The full length coding sequence was obtained in the form of an SphI/SmaI fragment inserted into plasmid pUC19 (fig. 3.0). The insert comprised the complete coding sequence for the 162 amino acid mature polypeptide, its own 18 amino acid leader sequence as well as 83bp of upstream and 30bp of downstream noncoding DNA. The DNA had to be manipulated to allow its insertion into a yeast expression vector. In yeast, there do not seem to be the same stringencies regarding the distance between promoter elements and the begining of the coding sequence with the levels of expression as those found in *E.coli*. However, it was still considered to be advantageous to have the initiation of translation site as close to the promoter as possible. As much upstream DNA as possible was removed. This procedure had to be carried out in two stages because any convenient flanking restriction sites also occurred within the coding sequence. The plasmid was digested with PstI and SmaI to give a 343 bp SmaI/PstI fragment encoding the C-terminal portion and a 233 bp PstI fragment encoding the N-terminal portion both of which were then purified. The SmaI/PstI fragment was then subcloned into plasmid pK18 followed by the PstI fragment (fig. 3.1). The correct copy number and orientation of the latter step was verified by separate StuI and AvaI digests. Plasmid pK18 is similar in size and sequence to pUC19 but carries a different antibiotic resistance gene. This alternative screening avoided complications in restriction mapping arising from possible

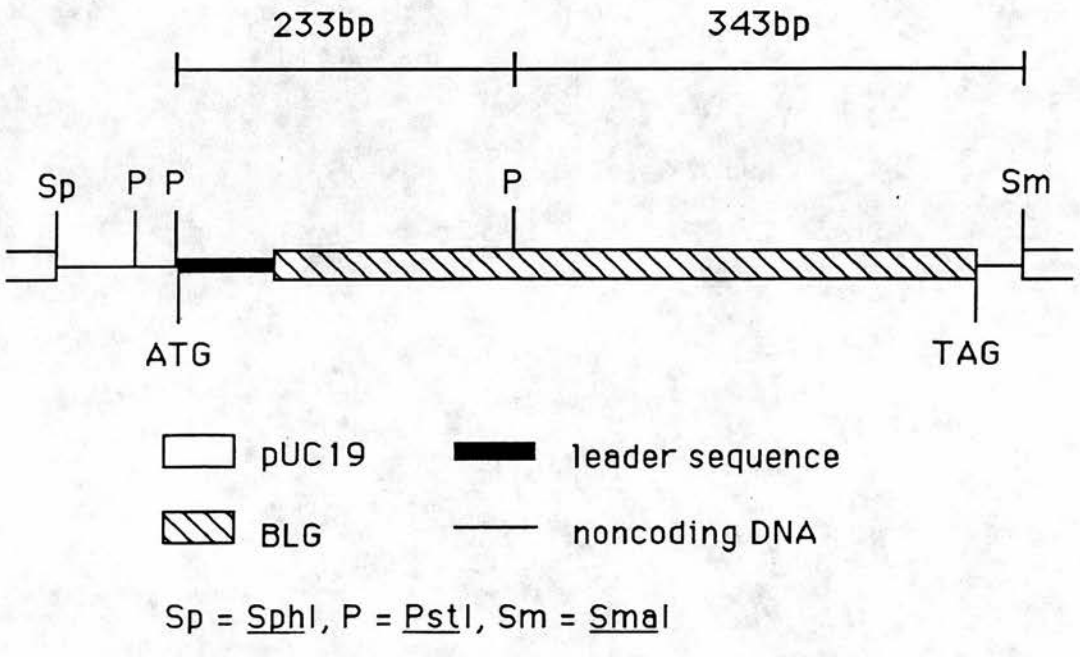


Figure 3.0 Diagram of pUC19BLG

Figure 3.1 Subcloning the BLG gene into plasmids pK18 and pUC1813.

1) Digest pUC19BLG with PstI and SmaI.

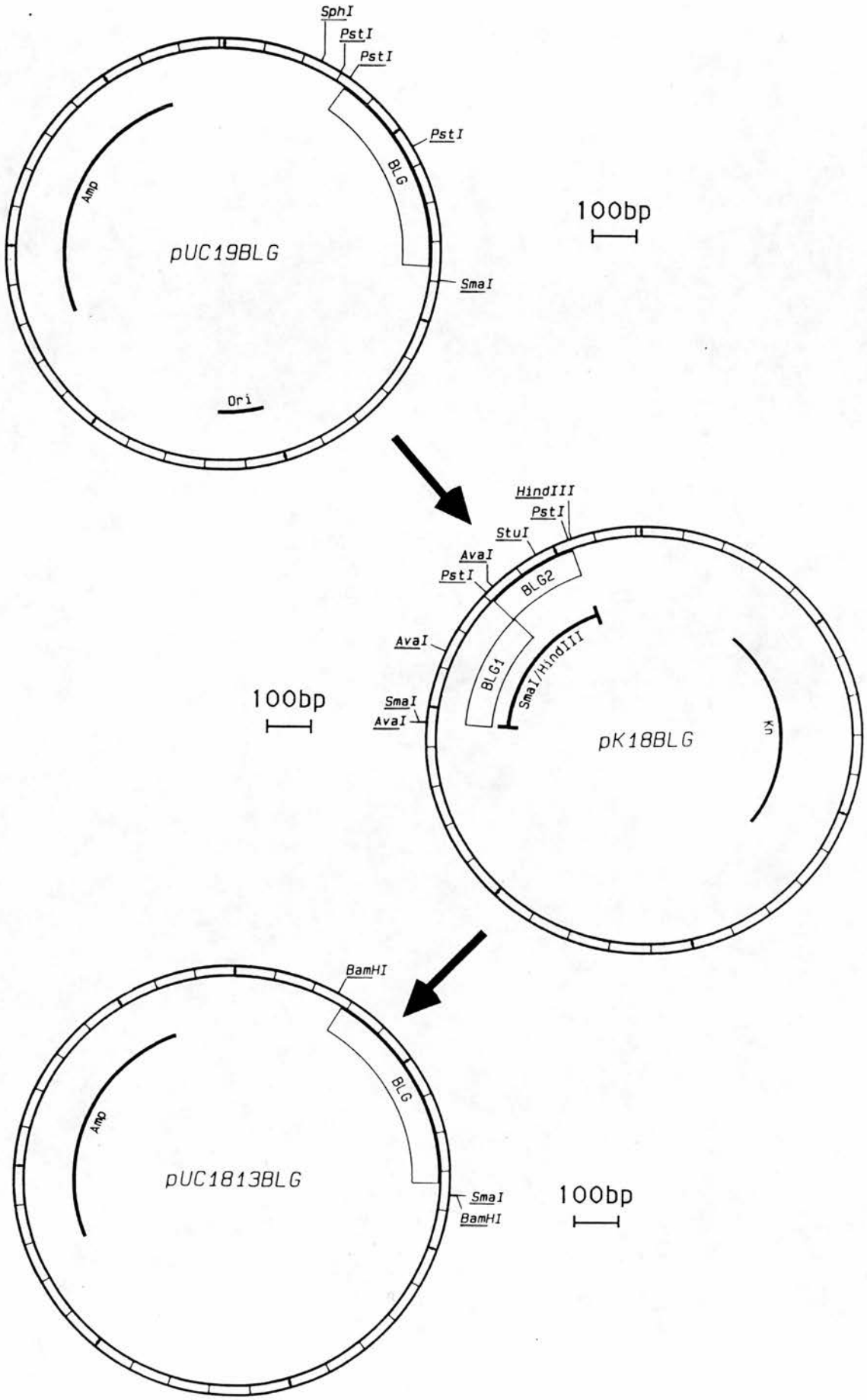
2) Purify 233bp PstI fragment and 343bp PstI/SmaI fragment.

3) Subclone PstI/SmaI fragment into pK18 followed by the PstI fragment to form pK18BLG.

4) Digest pK18BLG with HindIII and SmaI.

5) Blunt-end the HindIII end of the insert with Klenow fragment.

6) Subclone the blunt-ended insert into the SmaI site of pUC1813 to form pUC1813BLG.


SphI
PstI
PstI
PstI
BLG
Amp
pUC19BLG
SmaI
Ori
100bp
HindIII
PstI
StuI
AvaI
PstI
BLG2
AvaI
BLG1
SmaI/HindIII
100bp
SmaI
AvaI
pK18BLG
Km
BamHI
BLG
Amp
pUC1813BLG
SmaI
BamHI
100bp

contamination by the parent plasmid. As well as removing 80bp of upstream DNA, the complete coding sequence could now be moved about as a single unit by means of a HindIII/SmaI digest. The single stranded DNA overhang at the HindIII end of this fragment was treated with the Klenow fragment of DNA polymerase to form a double stranded "blunt end", from here on termed blunt-ending, and the whole fragment was subcloned into the SmaI site of plasmid pUC1813 (fig. 3.1). This latter subcloning step was purely a means of providing BamHI restriction sites close to both ends of the insert and as a result allowed convenient insertion into the expression vector.

A low copy number plasmid with an inducible promoter was initially used as the expression vector since the possibilty existed that BLG expression would prove toxic to the cell. The BamHI fragment from pUC1813BLG was inserted into plasmid pBM150 (fig. 3.2), an autonomously replicating, centromere-containing yeast shuttle vector that contains the URA3 gene for selection in yeast and the ampicillin gene for selection in *E.coli* as well as an 800 base pair region located between EcoRI and BamHI sites that contains the promoters for the GAL1 and GAL10 genes [157]. Cloning the insert into the BamHI site brought the expression of the BLG gene under the control of the GAL1 gene promoter. The resultant plasmid was called pBMBLG.

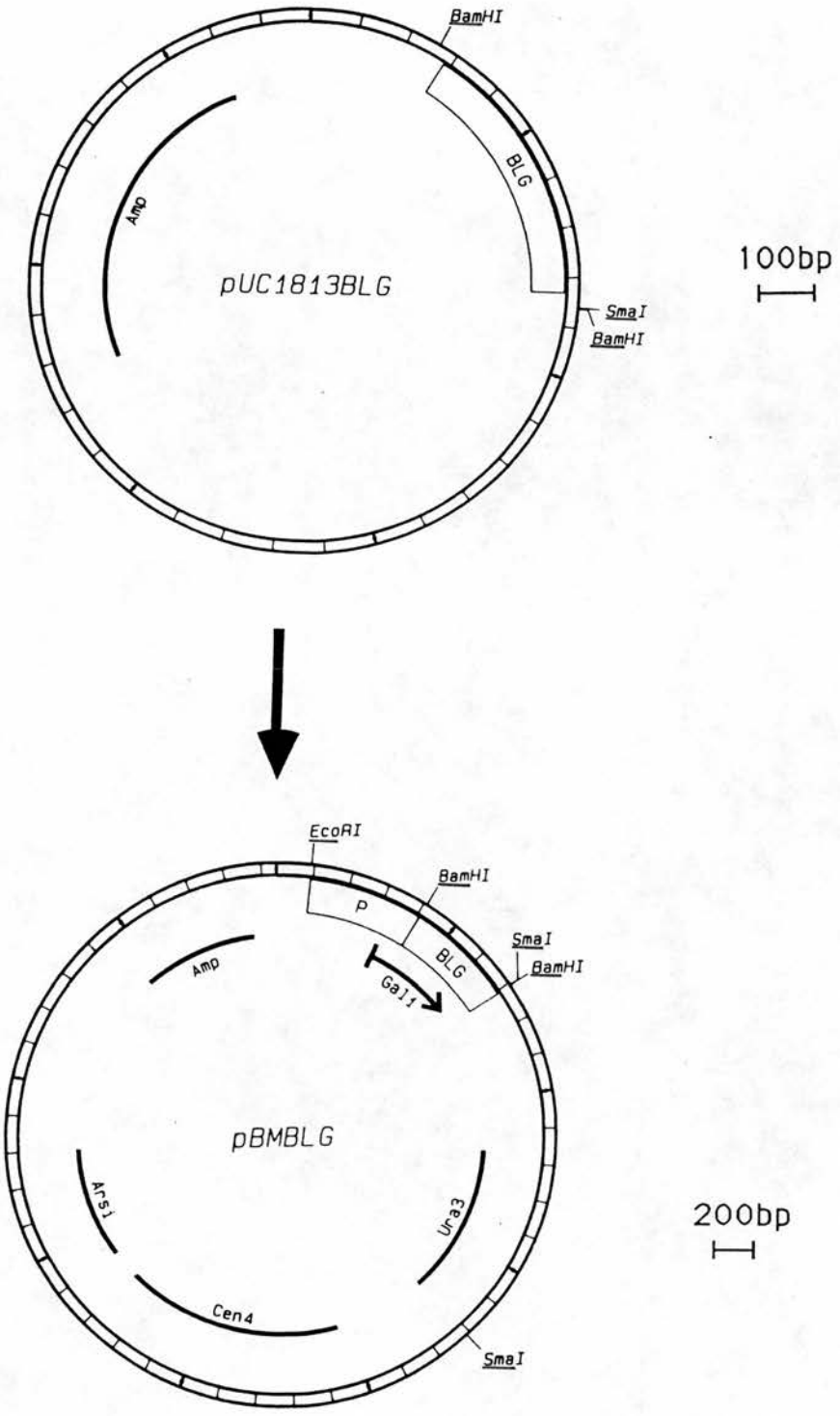


Figure 3.2 Insertion of the BLG gene into the expression vector pBM150.

1) Digest pUC1813BLG with BamHI.

2) Subclone insert into BamHI site of pBM150 to form pBMBLG.

3) Verify correct insertion by EcoRI and SmaI double digests.

3.2 Expression of BLG

Plasmids pBM150 and pBMBLG were transformed into yeast strain JRY188 and grown on YOM URA- plates. As already mentioned, these vectors rely on an inducible GAL promoter to control expression, the mechanism of which is briefly outlined below.

Galactose, when used as a carbon source, will be converted to glucose-6-phosphate by enzymes of the Leloir pathway encoded by the GAL genes. The GAL4 protein binds to sites upstream of each gene and activates transcription. In the presence of glucose, the GAL80 protein binds to the GAL4 protein thus preventing transcription. In the presence of galactose, an unidentified inducer molecule prevents the GAL80 protein from inhibiting the function of the GAL4 protein. Glucose, when present, acts as a catabolite repressor and any expression from the GAL genes is switched off, even when the carbon source is present as a mixture of glucose and galactose.

The following small scale experiment was set up to look for BLG expression. Different carbon sources were used to determine:

A) if continued growth in galactose was toxic

B) variations in BLG yield, if any, in cells grown originally in non-repressive and repressive carbon sources such as glycerol and glucose, respectively.

Exp.no.	Starter culture	2nd culture
1	2% Galactose---> o/n	2% Galactose---> o/n
2	3% Glycerol----> o/n	2% Galactose---> o/n
3	2% Glucose-----> o/n wash	2% Galactose---> o/n
4	2% Glucose-----> o/n	2% Glucose-----> o/n

Yeast transformed by pBM150 and pBMBLG were grown in 5ml of YP medium containing the different carbon sources overnight. A small aliquot was removed and grown overnight in 5ml of fresh medium whereupon the cells were then harvested and lysed. Proteins that had been secreted into the medium were precipitated with trichloroacetic acid. Lysate samples were then analysed by SDS-PAGE and stained with Coomassie Blue. Medium samples were also run but the more sensitive silver staining procedure was used. In this experiment cultures were grown in nonselective medium since, once transformed, centromeric plasmids are supposed to be highly stable [158].

The recombinant pBMBLG, if expressed, did not have any toxic effect as cells gave the same gross appearance of cell density in galactose as in glucose. SDS-PAGE of cell lysates failed to reveal the presence of any extra bands from the recombinant over the non recombinant strains. If BLG was being efficiently secreted, then the amounts of intracellular protein would be minimal and so the possibility existed that its presence was being masked by cellular

proteins. However, the silver stained gel of the medium samples failed to reveal any bands with a molecular mass comparable to that of BLG.

The samples were once again run on SDS-polyacrylamide gels along with 200ng of bovine BLG as a positive control. The presence of BLG was determined by Western analysis using a polyclonal rabbit antiserum raised against bovine BLG. Only the bovine control produced a positive signal, albeit weakly. At this point, it was realised that the acid stable BLG may not have been precipitated by trichloroacetic acid. 500ug of bovine BLG was added to 5ml of medium and treated with trichloroacetic acid. A sample was run on an SDS-polyacrylamide gel, no BLG was detected by Coomassie staining using this procedure. Another method would have to be used to detect the protein.

Ammonium sulphate precipitation was carried out on YPD medium containing BLG. Ammonium sulphate was added to 30% saturation and stirred gently at 20°C. At first the solution became characteristically cloudy but gradually cleared over a short period of time. This also occurred at 60% and full saturation. Loss of turbidity of the solution coincided with the appearance of a sticky residue on the magnetic stirrer, the amount of which increased with ammonium sulphate concentration. Any prospect of separating BLG from such a mixture seemed poor. This did not seem a plausable procedure for precipitating proteins secreted into the medium.

Further silver staining analysis of medium samples concentrated by ultrafiltration in Amicon systems and freeze drying also proved to be unsuccessful. Concentrated medium is viscous and its complex mixture produces unusual effects on gels such as highly erratic band patterns and the lanes stain as a brown smear with some protein bands staining a darker brown and others appearing as unstained areas contrasting against the background.. By these methods, it was impossible to determine whether there was any BLG present in any of the media. Trichloroacetic acid precipitated medium failed to produce these unusual effects, however, no BLG was detected using this procedure. It was possible that the acid treatment failed to precipitate the factors responsible for these anomalies and as a result produced cleaner gels.

The following experiment was set up to compare cells grown in selective YOM and non selective YPD media since:

A) it was possible that the "stable" plasmid was not being maintained.

B) YOM medium contains far fewer constituents and would perhaps provide cleaner gels.

Cells transformed with plasmids pBM150 and pBMBLG were grown in YOM and YPD, harvested, lysed and analysed by SDS-PAGE. Once again no BLG bands were detected by Coomassie staining. YOM medium from both pBM150 and pBMBLG cultures was dialysed overnight in 1xTBS, freeze dried and finally analysed by SDS-PAGE. Two other medium batches from pBM150 cultures were treated identically, however one was spiked with BLG before dialysis and the other was spiked just

before loading onto the gel. One other lane consisted of concentrated undialysed YPD that also contained BLG. The gel was first stained with Coomassie Blue and then silver stained (fig. 3.3). Only lanes spiked with BLG gave positive results. This gel shows some of the problems associated with silver staining medium extracts. The lanes stained well with Coomassie Blue however after silver staining, the proteins stained both dark and light. The YPD lane (lane 5) shows some of the swelling and erratic banding discussed earlier. Although the BLG in this lane stains well with Coomassie Blue it is very difficult to see it with the silver stain. This gel also showed that BLG is not being degraded to any great extent in the medium since the sample spiked before dialysis appears as a large well defined band.

3.3 New expression vectors

It was still possible that BLG was being produced in very small amounts as yet undetected. Two multicopy expression systems were used in the hope of amplifying the amount of protein.

Plasmid pVT102u is a multicopy shuttle vector that, along with the various selection genes, contains a multiple cloning site located between the promoter and downstream control sequences of the yeast ADH1 gene [159]. The BamHI/SmaI BLG fragment from pUC1813BLG was inserted into the BamHI/PvuII site of the vector to form pVTBBLG (fig. 3.4).

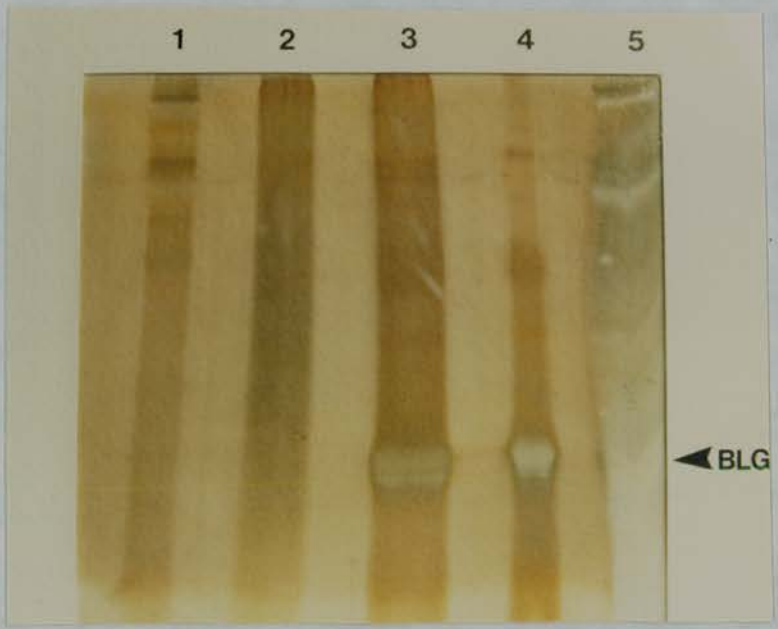


Figure 3.3 Silver stained SDS-polyacrylamide gel of concentrated medium supernatants.

1. YOM + pBMBLG
2. YOM + pBM150
3. YOM spiked with BLG before dialysis
4. YOM spiked with BLG just prior to SDS-PAGE
5. YPD spiked with BLG

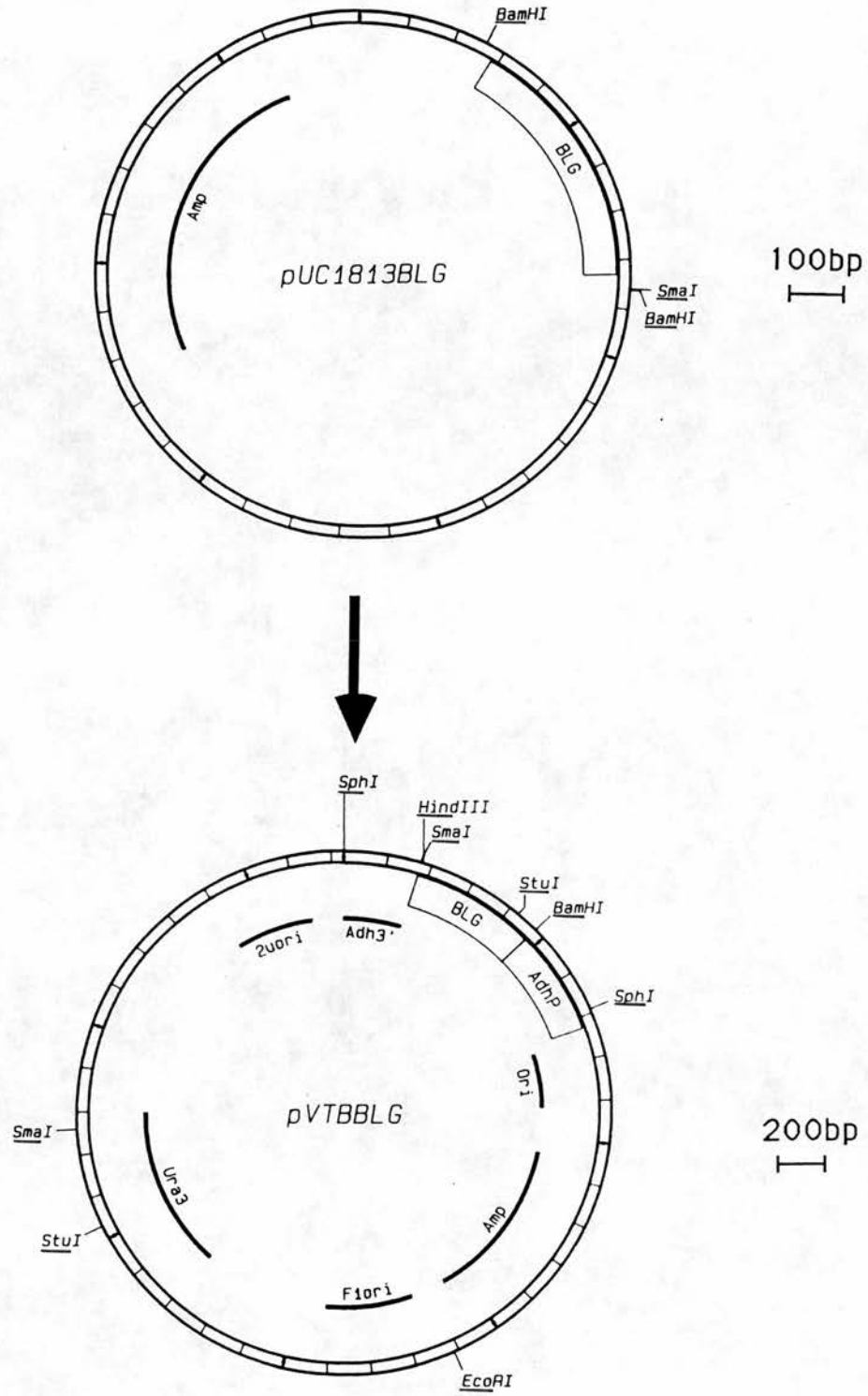


Figure 3.4 Insertion of the BLG gene into the expression vector pVT102u.

1) Digest pUC1813BLG with BamHI and SmaI.

2) Subclone insert into BamHI/PvuII site in pVT102u to form pVTBBLG

The second system was a hybrid of pBMBLG and the vector above. The promoter and down stream sequences from pVT102u were removed by SphI digestion and discarded. The remaining plasmid DNA was blunt-ended with T4 DNA polymerase. The EcoRI/SmaI fragment containing the GAL1/GAL10 promoter region and the BLG coding sequence from pBMBLG was purified and blunt-ended with Klenow fragment from DNA polymerase and inserted into the vector to produce a galactose inducible multi copy expression plasmid called pVTSBLG (fig. 3.5).

In the above two systems, the correct copy number and orientation of inserts was determined by restriction analysis.

PVTSBLG was transformed into yeast strain JRY188, and pVTBBLG was transformed into strain DBY747. Both were plated out on YOM URA- plates and transformants were analysed by plasmid rescue and restriction mapping.

Constitutively expressed plasmids pVT102u and pVTBBLG were grown overnight in 1ml of YOMglu. Galactose inducible plasmids pBM150, pBMBLG and pVTSBLG were grown in YOMgal. The cells were harvested, lysed and analysed by SDS-PAGE; the medium was dialysed against 1xTBS at pH7, freeze dried and half of each sample was analysed in by SDS-PAGE. Once again no extra bands corresponding to BLG were visible in any recombinant lanes.

Cellular extracts and the rest of the medium samples were blotted onto nitrocellulose along with BLG controls at differing concentrations using the Bio-Rad dot blot apparatus. Rabbit anti-

Figure 3.5 Generating a multicopy inducible expression vector, pVTSBLG.

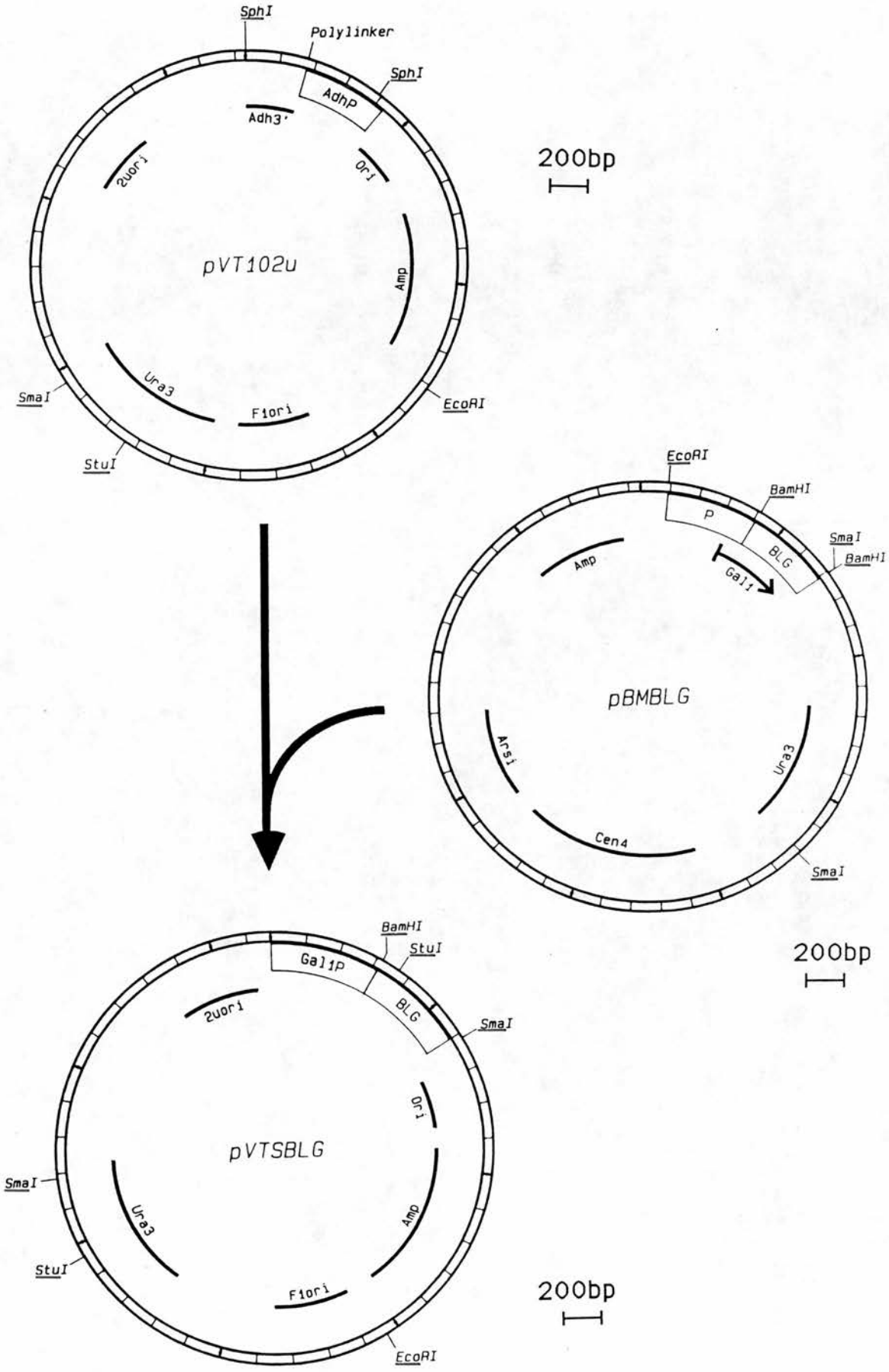
1) Digest pVT102u with SphI.

2) Discard the small SphI fragment containing the control elements and blunt-end the remaining plasmid DNA with T4 DNA polymerase.

3) Digest pBMBLG with EcoRI and SmaI.

4) Blunt-end the EcoRI end of the purified fragment, containing the GAL1 promoter and the BLG gene, with Klenow fragment.

5) Blunt-end ligate the insert into the remaining plasmid DNA to form pVTSBLG.


SphI
Polylinker
SphI
AdhP
Adh3'
2uori
Ori
pVT102u
Amp
Ura3
Fiori
SmaI
StuI
EcoRI
200bp
EcoRI
BamHI
P
BLG
SmaI
BamHI
Amp
Gal1
pBMBLG
Arsi
Ura3
Cen4
SmaI
200bp
BamHI
StuI
Gal1P
BLG
SmaI
2uori
Ori
pVTSBLG
SmaI
Ura3
Amp
StuI
Fiori
EcoRI
200bp

BLG antiserum was used to detect the presence of BLG but results were negative apart from the controls. However, controls at concentrations of less than 100ng failed to develop which may suggest a lack of sensitivity of the antisera or some factor concerning development.

At this stage two possibilities existed:

A) the expression was so small as to have escaped detection

or B) the cells were not producing BLG.

Increasing the culture size from 5ml to, for example, 1 litre, might reveal the presence of the protein. However, apart from the problems associated with scaling up the procedures, especially concentrating the medium from so many samples, it was felt that if the yield was so low using these expression systems, unmanageable volumes of cultures would have to be grown to provide enough protein for adequate analysis. Another method would have to be used to optimise expression of BLG.

Up until now there was no evidence of BLG being synthesised let alone secreted. Although this problem might have been solved by scaling up the experiments, the possibility existed that, for one of a variety of reasons (see later), the mature protein was not being synthesised. However, some light was shed on the matter by checking the preliminary subcloning steps with sequence constructs in the VAX mini-computer. In plasmid pK18BLG (fig. 3.1) an SphI site existed between the HindIII and PstI sites, a fact not taken into consideration in the design strategy. This restriction site contained an ATG codon that was in frame with respect to the BLG

coding sequence. Initiation of translation would most likely occur at this upstream ATG resulting in the addition of four extra amino acids, Met-Pro-Ala-Ala, onto the front of the leader sequence. It was unclear how the addition of these amino acids onto the leader sequence would affect the final destiny of the protein as it had not even been ascertained whether yeast would direct secretion of BLG using its own leader sequence. In addition to this, the possibility also existed that, due to some cloning artefact, there was a mutation within the coding sequence. Assuming that the native leader would prove functional, it was decided that the short stretch of DNA encoding the extra amino acids would be removed and the complete DNA coding sequence checked for mutations.

3.4A Lack of expression - Possible causes

As already mentioned, no BLG protein was detected using the GAL1 and ADH1 promoters. There are several stages where expression and secretion of BLG may have failed.

If the promoters were not functional, then transcription of the gene would not have been initiated. To test this, the gene encoding some other protein commonly used in yeast expression studies, eg. B-galactosidase, could have been inserted next to the promoters. Successful expression could then be detected by simple enzyme assay. However, both these promoters successfully directed expression of Drosophila ADH in the same laboratory [160], which probably rules out failure at this particular stage.

The addition of the extra four amino acids has already been implicated in the breakdown of the pathway. This could be verified by excising the DNA encoding these amino acids and reinserting the genes into the expression plasmids to look for expression. A successful result to this experiment would still raise questions as to the precise problem caused by these amino acids. Problems may have arisen at the levels of transcription, translation and/or protein stability. The presence of specific transcripts could be determined by Northern analysis. Abundant levels of mRNA not resulting in detectable protein may point to problems during translation or protein stability whereas low yields of BLG mRNA may point to problems of transcription or mRNA stability. mRNA half-life is determined by the rate of transcription, mRNA stability and

the amount of mRNA removed as a result of translation. The BLG mRNA half-life can be determined by adding transcriptional inhibitors such as phenanthroline to an exponentailly growing culture. The abundance of BLG mRNA isolated at various time points can be determined by dot blot or Northern analysis using radio labelled BLG cDNA as a probe. If the mRNA half-life is extended in the presence of translational inhibitors such as cyclohexamide this may suggest that at least some of the mRNA turnover results from translation. If the half-life in the presence of cyclohexamide remains the same this may suggest that the problem may be one of transcript stability, which may be especially true for the GAL1 promoter directed transcripts which are probably not polyadenylated. However, care must be taken in making absolute conclusions since there is a complex interplay between translational efficiency and mRNA half-life that is poorly understood. As a result, possible aberrations arising from the use of these metabolic inhibitors must be taken into account.

Problems resulting from translation may be detected in polysome gradients. Fractionation of the gradient followed by dot blot analysis may show a lack of ribosome binding pointing to problems arising from initiation or premature termination of translation. Gradual degradation of the BLG protein could be followed in the presence of cyclohexamide. Detection of the desired protein would be accomplished by Western analysis using an antiserum specific for BLG. Loss of intracellular signal not paralleled by a concomitant increase in secreted BLG levels would suggest the occurence of

protein degaradation. The formation of insoluble aggregates cannot be ruled out. Treatment of the heavy membrane fraction with urea and 2-mercaptoethanol may solublise aggregated BLG.

Before carrying out these control experiments to resolve the above questions, it was decided to proceeed with the expression studies using one of the most powerfull yeast promoters inserted next to a BLG gene without the extra four amino acids in a final attempt to gain functional expression.

3.4B Sequencing the BLG gene

The SphI/SmaI fragment from pK18BLG was purified and blunt-ended using T4 DNA polymerase. This insert was then subcloned into pUC1813 to provide BamHI restriction sites. This plasmid was called pUC1813TBLG. From there, the BamHI fragment was removed and inserted into bacteriophage M13mp18 for sequencing. Since inserts were obtained in both orientations, the full insert could be sequenced using the universal primer. Sequence results showed three changes from the expected sequence. However, none of these changes would affect the transcription or translation of BLG.

A) There was a G-> T substitution in the eleventh codon of the mature polypeptide. The full coding sequence from plasmid pUC19BLG had originally been constructed in another laboratory from genomic

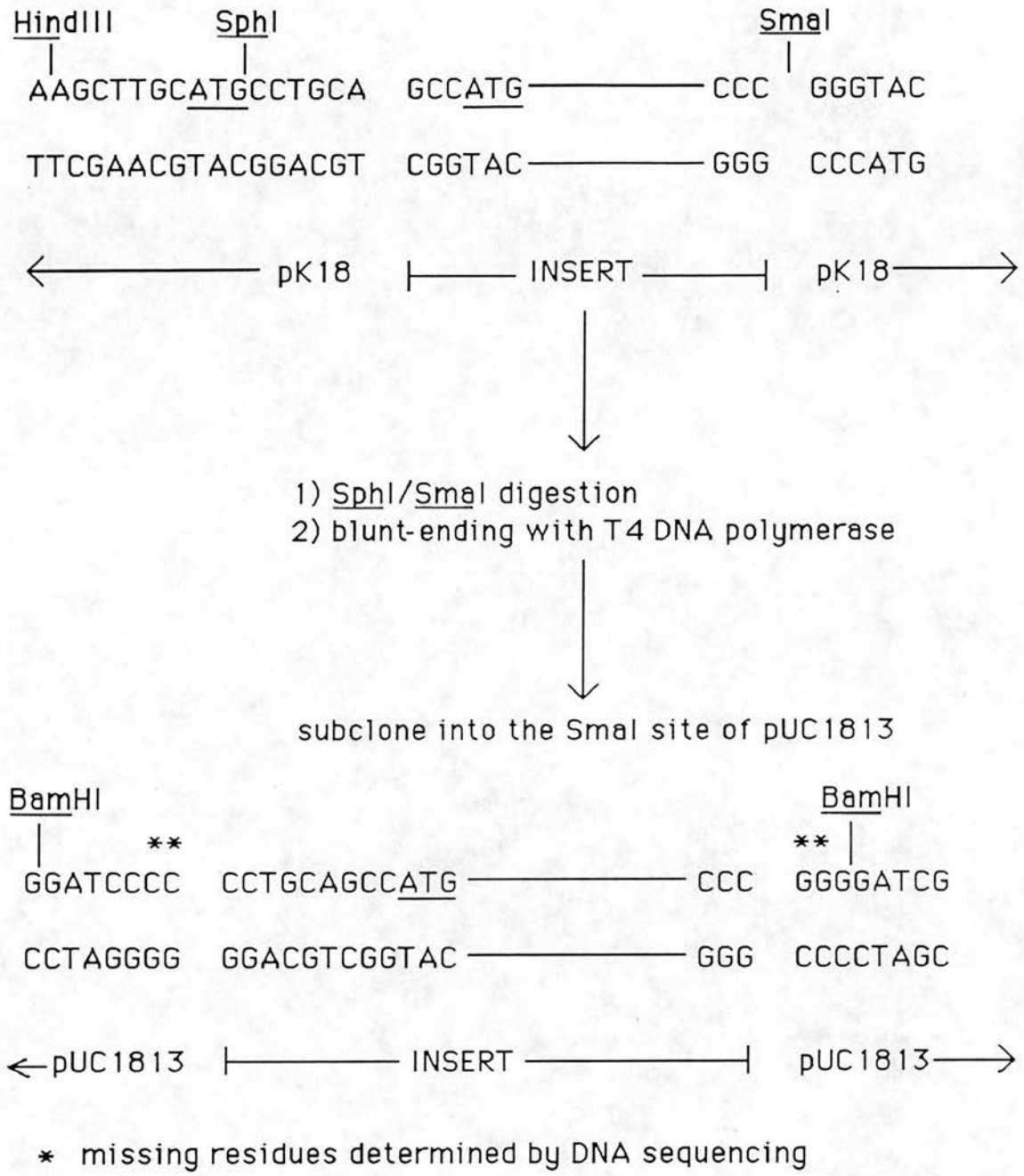
DNA and cDNA. This mutation was present as a design error in the oligonucleotide used to link the two fragments. Fortunately, this was a silent mutation and so failed to change the amino acid sequence.

B) There were two artefactual mutations outside the coding sequence (fig. 3.6).

The cloning procedure, involving the excision of the upstream ATG, resulted in the loss of two C residues from the noncoding upstream end and two G residues from the noncoding downstream end. The loss of these residues cannot be explained by a lack of detection arising from stacking artefacts on sequencing gels as many similar strings of residues were always distinguishable. It is impossible to determine whether the two C residues were lost from the insert or the vector pUC1813. However, since the two G residues were lost from the vector, it is likely that there was limited degradation of the SmaI digested vector resulting in the loss of these residues. The cause must remain unknown. The evidence from the sequence data was further supported when the plasmid, once tested, failed to be cleaved by SmaI.

3.5 Expression using plasmid pMA91

The DNA sequence had now been checked and the short stretch of DNA coding for the extra amino acids removed. The question now arose as to whether the extra amino acids had caused the protein to be degraded, or whether any lack of expression was a result of the



* missing residues determined by DNA sequencing

Figure 3.6 Mutations generated outwith the coding sequence during removal of the upstream ATG.

expression systems being in some way unsuitable. Other studies within the group, involving the expression of Drosophila ADH using the same vectors and the same yeast strains failed to detect expression at the protein level [160]. The protein was finally isolated when a different yeast strain was used to conduct the experiments.

It was decided to use a different yeast strain together with a different expression plasmid which contained one of the strongest known yeast promoters.

Plasmid pMA91 is a multicopy yeast shuttle vector that contains the LEU2 gene for selection in yeast and the ampicillin gene for selection in *E.coli*. The plasmid contains the PGK promoter as well as the PGK downstream control sequences and has been used successfully for the high level expression of calf prochymosin [161]. The BamHI fragment from pUC1813TBLG was inserted into the BglII site next to the promoter, destroying the cloning site in the process, to form pMABLG (fig. 3.7). Recombinants were mapped with HindIII/PvuII and EcoRI/PvuII double digests to ensure single copy insertion in the correct orientation. Plasmids pMA91 and pMABLG were both transformed into yeast strain AH22 and plated out on YOM LEU- plates. Transformants were analysed by plasmid rescue and restriction mapping.

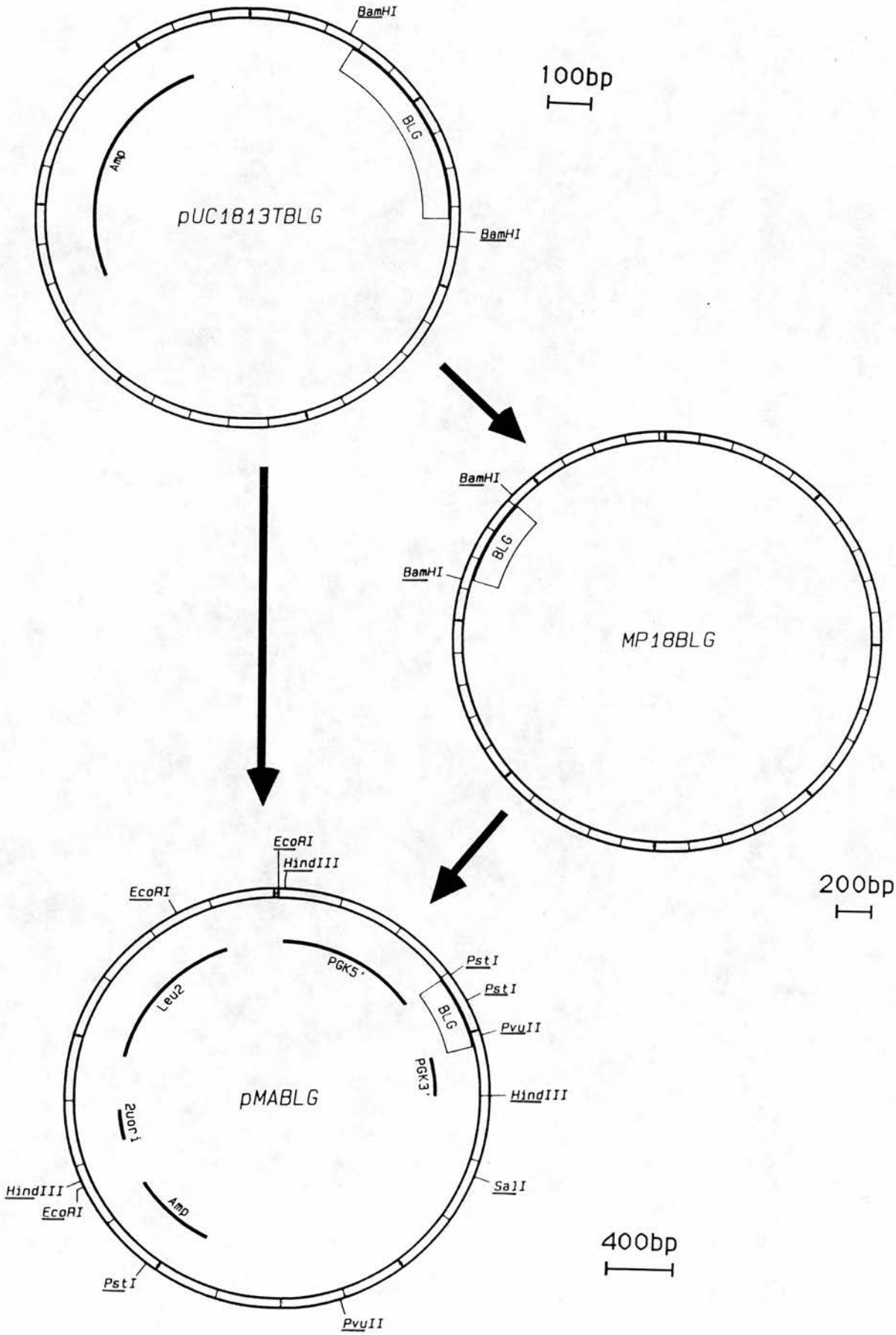
Cells transformed with plasmids pMA91 and pMABLG were grown overnight in 10ml of YOM LEU- medium. This starter culture was then added to 100ml of the same medium and grown for a further 24

Figure 3.7 Insertion of the BLG gene into the expression vector pMA91.

1) Digest pUC1813TBLG with BamHI.

2) Subclone the insert into the BglII site of pMA91 to form pMABLG.

For sequencing and site-directed mutagenesis, the BamHI fragment from pUC1813TBLG was inserted into bacteriophage M13. Mutant genes were subsequently subcloned into pMA91.


BamHI
100bp
BLG
Amp
pUC1813TBLG
BamHI
BamHI
BLG
BamHI
MP18BLG
200bp
EcoRI
HindIII
EcoRI
PstI
PstI
PvuII
PGKS'
Leu2
BLG
PGK3'
pMABLG
HindIII
2ori
SalI
HindIII
EcoRI
Amp
PstI
PvuII
400bp

hours. The cells were harvested, and lysates were analysed by SDS-PAGE and stained with Coomassie Blue (fig. 3.8A). No BLG bands were detected in the cell extracts, although, the possibility existed that its presence was being masked by cellular protein bands.

The medium was concentrated from 100ml to 8ml using an Amicon ultrafiltration cell and then dialysed against 20mM sodium phosphate at pH6.5 overnight. After dialysis, the medium was concentrated in an Amicon microconcentrator to 300ul. Concentrated medium samples of 2.5ul from recombinant and non recombinant cells were analysed by SDS-PAGE and stained with Coomassie Blue (fig. 3.8B). There were striking differences between the two samples. Bands the size of BLG were present in the recombinant lane that were totally absent in the non recombinant lane. Both lanes exhibited identical band profiles which represented other proteins common to both medium samples. It was more than likely that these bands in the recombinant lane resulted from the presence of BLG. 30ul of this concentrated sample was analysed by reverse phase h.p.l.c. and produced the elution profile shown in fig. 3.9. The major peak was collected for sequencing. The first 10 amino acids were determined (fig. 3.10) and gave the following sequence:

Ile-Ile-Val-Thr-Gln-Thr-Met-Lys-Gly-Leu

The amino acid sequencing was kindly performed by Linda Kerr.

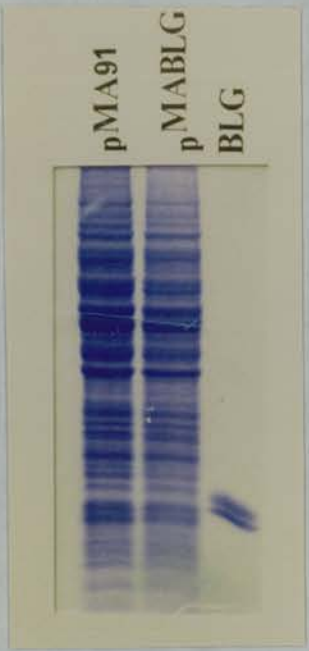


Figure 3.8A SDS-PAGE of cellular extracts of cells transformed by pMA91 and pMABLG.

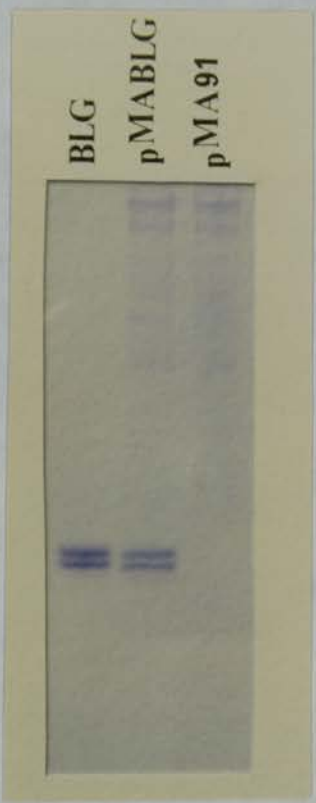
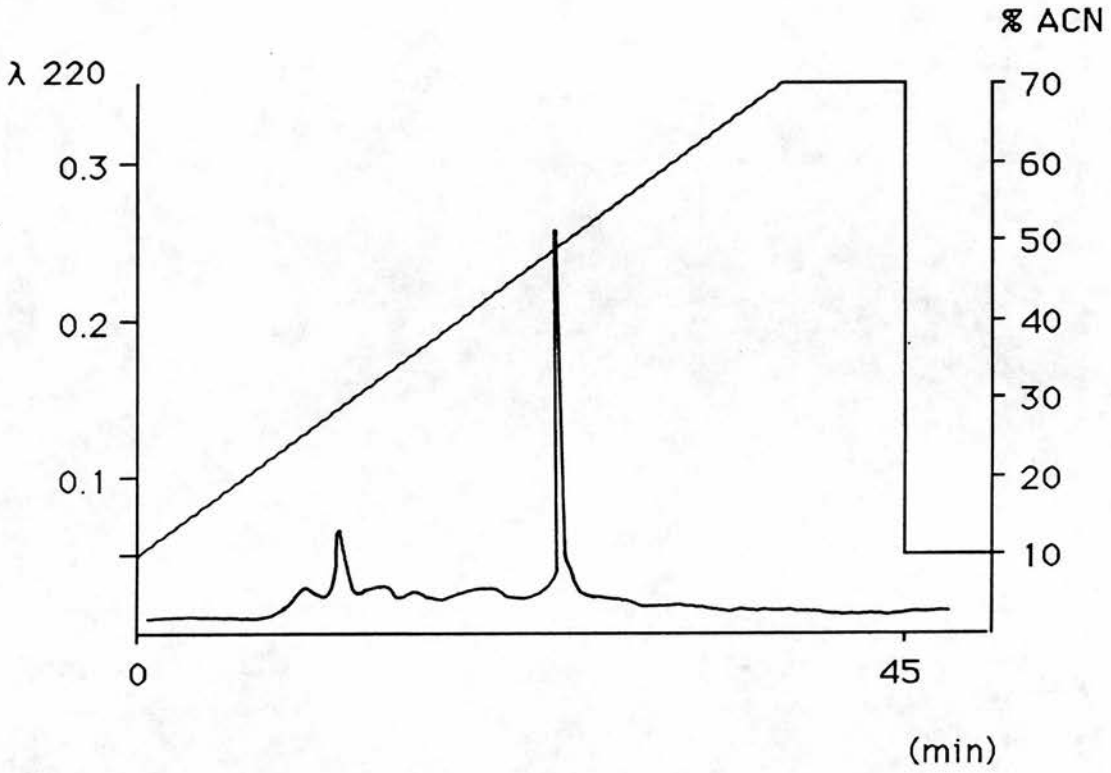


Figure 3.8B SDS-PAGE of concentrated medium supernatants. Amount of BLG in control lane is approximately 7-8ug.



buffer A: 0.1% TFA in water
buffer B: 0.08% TFA in 70% ACN

TFA = trifluoroacetic acid
ACN = acetonitrile

Figure 3.9 Reverse phase h.p.l.c. profile of concentrated medium.

cycle	1	2	3	4	5	6	7	8	9	10
Ala	16.7	4.6	3.7	8.4	0.5	-1.4	-0.3	0.4	1.5	0.2
Arg	1.8	0.9	3.0	-1.0	-0.2	-0.1	-0.4	0.4	0.2	0.1
Asn	0.5	-0.3	0.5	-0.4	-0.7	-0.4	-0.1	0.6	0.4	-0.2
Asp	16.1	-0.6	1.0	0.2	-1.0	0.6	6.3	6.8	4.7	-0.1
Cys	-1.0	-1.0	-1.0	-1.0	-1.0	-1.0	-1.0	-1.0	-1.0	-0.1
Glu	1.9	-1.6	-1.5	0.7	64.9	1.5	3.1	0.2	1.6	-1.3
Gln	-0.4	-0.1	-0.2	5.7	**281.3**	1.9	1.5	-1.9	2.9	-2.7
Gly	6.2	3.5	-0.4	-2.1	-2.0	4.6	-1.6	0.7	**239.8**	0.0
His	0.3	0.0	-0.1	-0.1	0.2	-0.2	-0.4	-0.1	0.5	0.0
Ile	**460.3**	**410.2**	0.0	0.7	6.6	0.0	-1.3	-0.2	0.1	0.7
Leu	6.4	3.2	1.0	-0.2	-0.9	-0.6	1.7	0.0	0.6	**263.8**
Lys	-0.4	-0.4	0.3	-0.1	1.8	-0.4	-0.9	**290.4**	2.7	0.8
Met	0.4	-0.3	0.4	0.3	-0.3	-1.0	**306.0**	0.6	1.5	0.5
Phe	1.8	0.5	0.2	0.0	-0.4	-1.0	-0.7	0.6	0.7	1.6
Pro	0.8	-1.2	-0.6	4.0	6.0	1.2	5.1	4.3	0.9	1.3
Ser	2.6	0.4	0.2	-0.9	1.1	-0.4	0.5	1.5	0.4	-0.3
Thr	0.7	0.0	-1.4	**265.0**	0.0	**212.2**	2.7	1.6	0.2	-1.2
Trp	4.7	-0.3	2.9	-0.3	0.8	0.8	-1.2	3.3	0.0	0.1
Tyr	-0.5	1.4	0.5	0.6	-0.6	-0.2	0.3	1.4	0.5	-0.6
Val	0.5	3.7	**369.2**	0.2	-0.7	-0.8	0.3	0.5	0.2	4.8

Figure 3.10 N-terminal amino acid sequence analysis. Lag corrected tabulation, in pmols, of the amino acids released by 10 cycles of Edman degradation.

This agrees with the predicted sequence of the mature ovine BLG protein obtained from the translation of the DNA sequence as well as that of the amino acid sequence for the protein [19].

These results show that recombinant cells synthesise ovine BLG which is successfully secreted into the surrounding growth medium. Comparisons between the band intensities on polyacrylamide gels with a known amount of bovine BLG standard showed an approximate yield of 6-8mg per litre of culture (fig. 3.8B). As well as confirming the identity of the protein, the amino acid sequence has shown that the protein has been correctly processed, with regard to its cleavage from the leader sequence, and is present in its mature form.

BLG on the denaturing gel was present as two bands of equal intensity. This cannot be readily explained. The BLG control is a mixture of bovine variants A and B and it is possible, though unlikely, that the slight difference in amino acid composition could lead to variations in the mobility of the two proteins in denaturing gels. However, this argument cannot be used for the ovine protein since it is synthesised from a single gene. This artefact is most likely caused by the incomplete unfolding of protein secondary structure perhaps as a result of insufficient reducing agent.

Identical amounts of bovine BLG were boiled for five and ten minutes in sample buffer containing fresh 2-mercaptoethanol. Another sample was boiled for five minutes in sample buffer lacking

2-mercaptoethanol. The samples were then run on an SDS-polyacrylamide gel and stained with Coomassie Blue (fig. 3.11). The proteins boiled in 2-mercaptoethanol were still present as two bands, even after ten minutes, but the higher molecular mass band was now more prominant. The protein boiled in the absence of 2-mercaptoethanol was present as a single band but migrated further, as expected, due to the presence of limited secondary structure provided by the non reduced disulphide bridges. There has been no report of this anomaly regarding BLG in the literature. This is not surprising since most of the electrophoretic analysis has involved the use of non denaturing conditions. Studies involving SDS-PAGE [162] have only revealed single bands. However, since the amount of BLG loaded onto these gels seems quite large, judged by the heavy band intensities, it is possible this doublet may be masked by overloading. Many subsequent gels have shown slight variations in BLG band profile but the one discussed here seems to be most prevalent. It is likely that the slower band is the fully denatured protein and the faster band has either lost some amino acids or has limited secondary structure. If this is so, it cannot be readily explained how identical conditions can produce two distinct populations of proteins.

3.6 Large scale preparation of BLG

Simple purification procedures of BLG from bovine milk have relied on properties of the native protein and have been well documented (see introduction). However, there were no guarantees that yeast would synthesise the protein in its structurally native form,

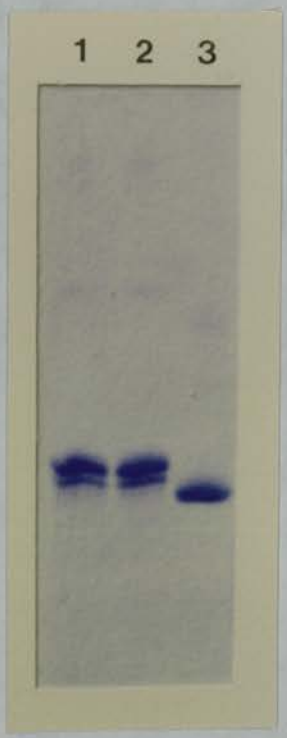


Figure 3.11 SDS-PAGE of bovine BLG

1. BLG boiled for 5 min + 2-mercaptoethanol.
2. BLG boiled for 10 min + 2-mercaptoethanol.
3. BLG boiled for 5 min - 2-mercaptoethanol.

enabling the use of such procedures. The possibility also existed that constituents in the medium may affect the behaviour of the protein in solution. A new protocol was devised in the hope of obtaining enough pure protein to characterise its solution properties.

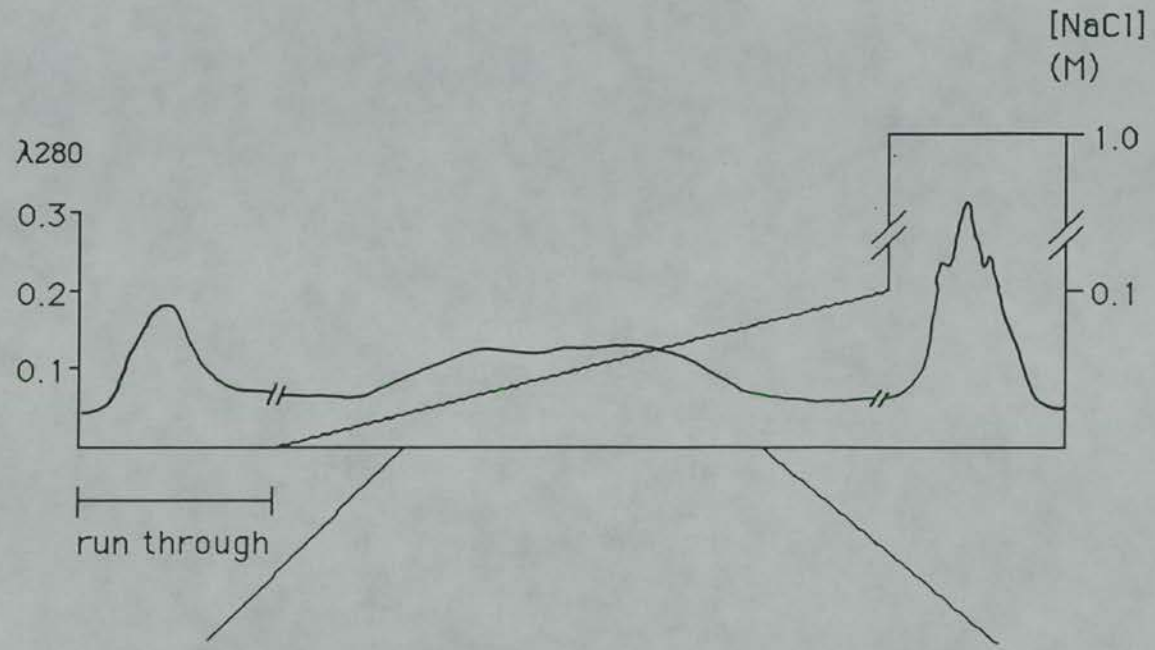
A 2.5 litre culture was grown to stationary phase in unbuffered YOM LEU- medium. The cells were removed by centrifugation and the medium was concentrated from 2.5 litre to 12ml at 4°C using Amicon ultrafiltration cells with YM10 filters which had a relative molecular mass (M_r) cut off of 10,000. During concentration, a precipitate gradually formed which was removed by centrifugation. It was unclear at this stage whether the precipitate was already present but too dilute to see in unconcentrated medium or whether it was a result of some concentration factor. The supernatant was subsequently dialysed overnight in 50mM sodium phosphate, pH6.2, at 4°C. The approximate protein concentration in the supernatant was determined by its relation, mg of protein/ ml = 1.11 x absorbance at 280nm [163]. There was approximately 24mg of protein in 12ml of supernatant. Gel electrophoresis showed BLG to be the most abundant protein present. It is interesting to note here that the pH of the medium at stationary phase was 2.94. The electrophoresis step mentioned above showed that BLG is soluble at this pH which may suggest the protein exists in its native form. However, SDS-PAGE of the precipitate resuspended in buffer showed that BLG is a major component. This aspect will be discussed later. The supernatant was then fractionated by ion exchange chromatography. The sample was loaded onto a 2cm x 5cm DEAE Sephadex A50 column

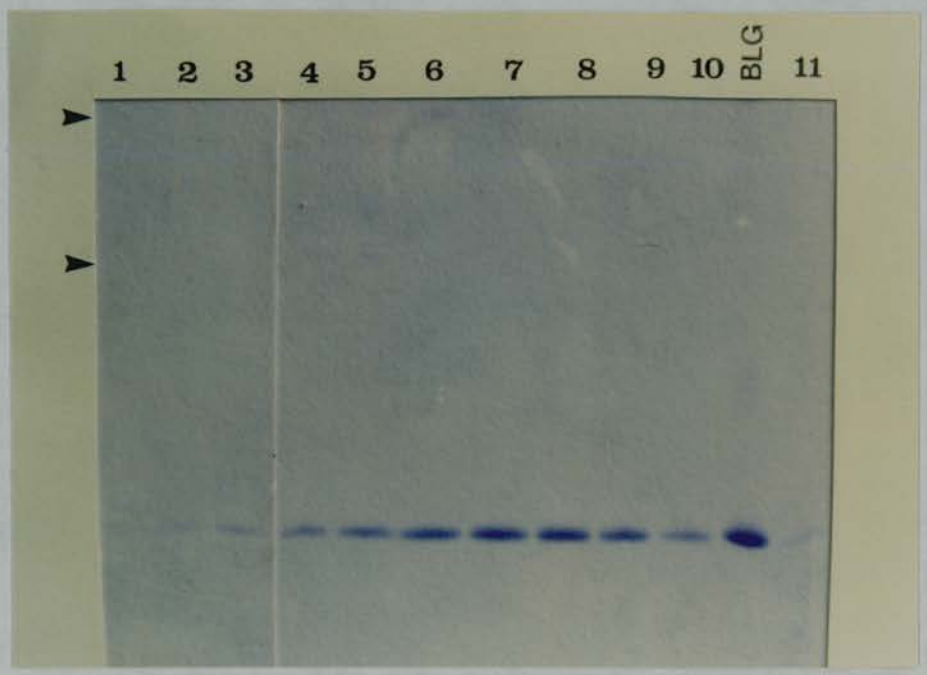
which was then washed with several column volumes of 20mM sodium phosphate buffer at pH 6.2. Protein was eluted from the column in a 0 to 0.1M sodium chloride gradient in 20mM sodium phosphate buffer at pH6.2. This was followed by a single step wash in 1M sodium chloride in the same buffer. The elution profile and the SDS-polyacrylamide gel of the gradient eluent fractions are shown in figure 3.12.

The elution profile showed some material eluting in the void volume during loading of the sample and a broad peak eluting from the gradient covering fractions 26 to 60 which may be an accumulation of several peaks. Gel electrophoresis showed that BLG was eluted throughout the range of the broad peak with only minor contamination of higher molecular mass bands. No BLG was detected in either the early peak, which represented material that failed to bind to the column, or in the peaks eluting in the 1M sodium chloride step. The bed height of the column decreased significantly during elution. As the sample was eluted by increasing ionic strength, it was then subjected to gel filtration effects of the shrinking column. This may account for BLG eluting as a broad peak, however, it is also possible the gradient was too broad. A different ion exchange matrix less susceptible to shrinkage would be utilised in future.

Ion exchange chromotography had removed many of the contaminants present in the sample and the ones remaining were thought large enough to be separated by gel filtration.

Figure 3.12 Elution of BLG from Sephadex A50.
Bed size: 2 x 5cm.
Eluent: 0 to 0.1M sodium chloride gradient in 20mM Sodium chloride buffer, pH 6.2, followed by a 1M salt wash.





SDS-PAGE of fractions collected from the broad peak.

Position of the arrows indicate higher molecular mass bands.

The fractions containing BLG were pooled and concentrated to a final volume of 5ml. The sample was then dialysed in several changes of distilled water, freeze dried and resuspended in 850ul of 50mM sodium phosphate buffer at pH6.2. Next it was loaded onto a 1.6cm x 94cm Sephacryl HS 200 column and eluted in the same buffer. Eluent fractions were analysed by SDS-PAGE which showed that BLG, eluted from the column, appeared to be pure (fig. 3.13A). The fractions were then pooled and concentrated. This supernatant contained dextran blue which had previously been used to estimate the void volume of the column. The supernatant was dialysed in distilled water then freeze dried. The precipitate was resuspended in 100ul of water and 2ul of this sample was analysed by SDS-PAGE (fig. 3.13B).

This purification procedure showed that BLG had eluted at the void volume suggesting the protein had formed high molecular mass aggregates which dissociated under the denaturing conditions during electrophoresis. It is possible that freeze drying the protein before chromatography had favoured the formation of such complexes. Freeze drying may not be a suitable procedure for concentrating BLG at this stage. This hypothesis is supported by comparing figures 3.13A and 3.13B. There are many bands present in figure 3.13B not present in figure 3.13A. These bands are possibly aggregates of BLG but some may represent contaminants too dilute to be seen in unconcentrated supernatant. However, the eluent from the column was cloudy which might have been a result of microbial contamination. The yield of protein, recovered from the column, was lower than expected. The ionic strength of the sodium

Figure 3.13A SDS-PAGE of BLG eluted from the Sephacryl HS200 column.

Figure 3.13B SDS-PAGE of freeze dried eluent from the Sephacryl HS200 column.

1. Freeze dried sample 2. BLG

phosphate elution buffer may not have been sufficiently strong to inhibit the formation of weak ionic interactions between the protein and the column matrix. That such interactions occurred was shown when the column was washed in 1M sodium chloride. As the buffer washed through the column, the leading front of the buffer gradually acquired dextran blue which finally eluted as a single band.

Many conditions used in this first attempt at large scale protein purification would be altered in subsequent procedures. However, it was felt more important to concentrate on making the protein mutants, discussed in the introduction, and delay refinement of the purification procedures until this had been achieved.

3.7 Site-directed mutagenesis of BLG

Mutant BLG genes were generated to answer some questions regarding the roles played by certain amino acids in determining the structure of BLG. Two methods were used to compare efficiency and ease of creating mutants: one was based on the Eckstein procedure outlined in the Amersham manual, and the other was the Kunkel procedure [164]. Two different orientations of insert in M13 were used as templates, as in the previous sequencing steps, so that each mutant could be verified by sequencing using the universal primer. The following mutations were made.

Cysteine 119 and 121 were converted to serine as separate mutants (fig. 3.14A)

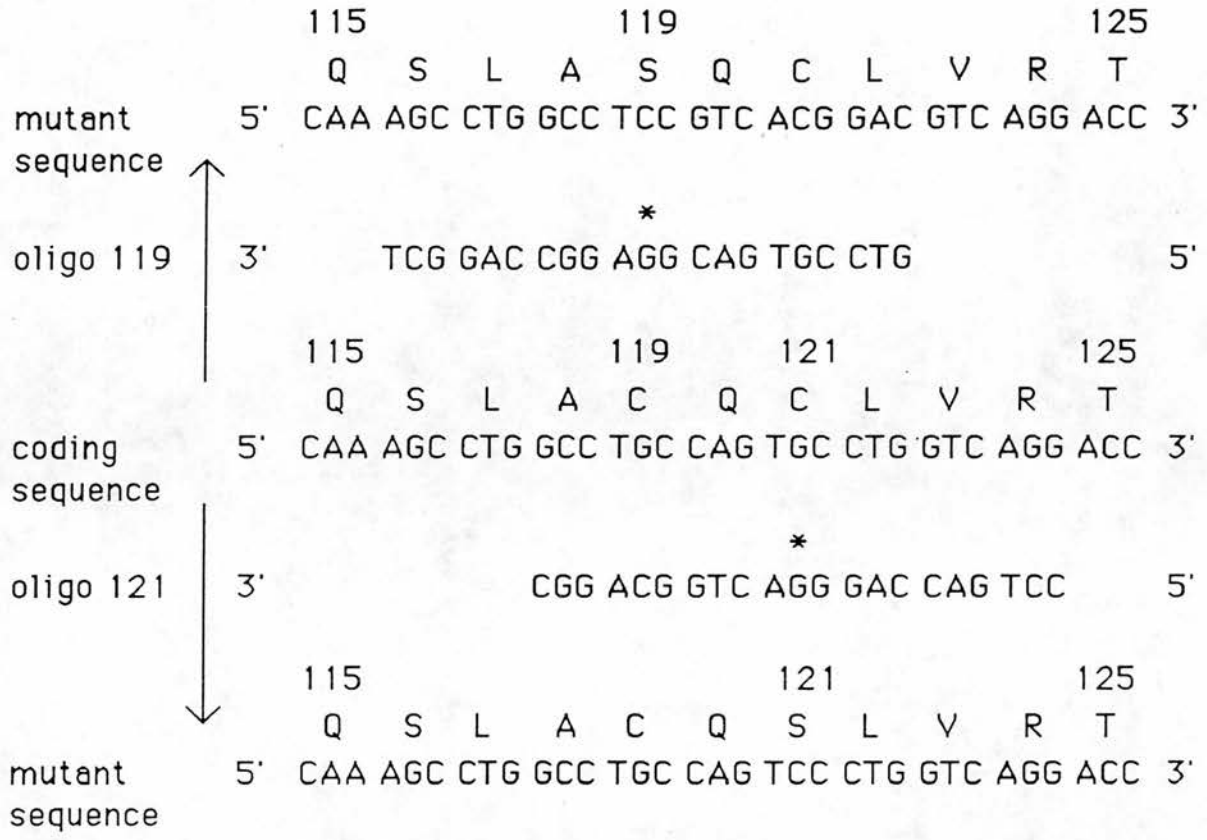


Figure 3.14A Oligonucleotides used to generate mutants 119 and 121.

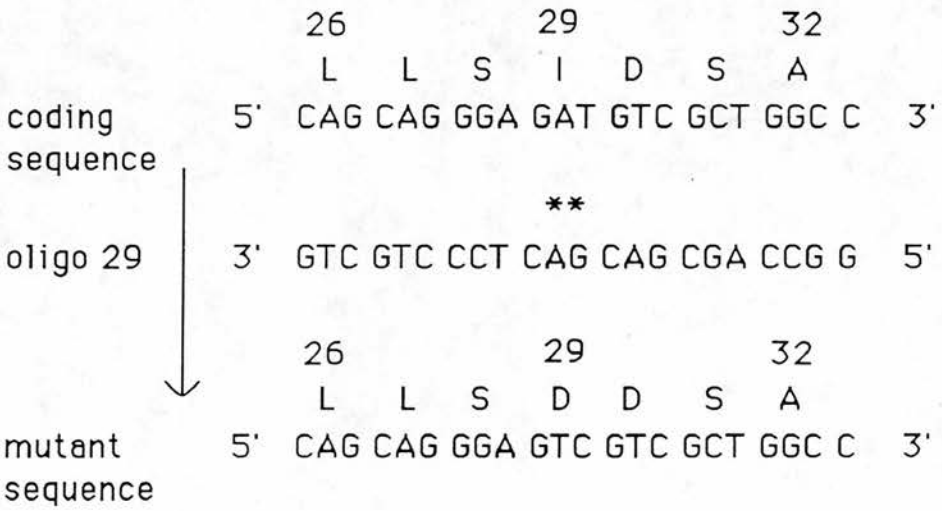


Figure 3.14B Oligonucleotide used to create mutant 29.

The creation of these mutants would hopefully settle the controversy regarding the position of one of the disulphide bridges discussed in the introduction.

Isoleucine 29 was changed to an aspartate (fig. 3.14B).

X-ray crystallography analysis of the BLG dimer indicated that this isoleucine on each monomer formed hydrophobic interactions at the dimer interface. The negative charge on the side chain of aspartate might provide enough repulsion to prevent dimerisation. The monomers would then prove suitable for NMR studies.

Lysine 70 was changed to an asparagine (fig. 3.14C).

This last mutant would be the first in a series to alter the residues involved in the hydrophobic pocket. The most up to date model has shown that lysine 70 is located at the entrance to the pocket and may be involved in binding the hydroxyl group in the isoprene tail of retinol. Substituting lysine with asparagine may affect binding of retinol to BLG and thus confirm the role of the hydrophobic pocket as the binding site.

Two plaques from each mutant transformation were sequenced. Six out of eight plaques proved to be mutant. One made by the Eckstein method was a wild type and one from the Kunkel method generated unreadable sequence as a result of a sequencing artefact. Both mutagenesis methods had a comparable efficiency and both were relatively easy to use.

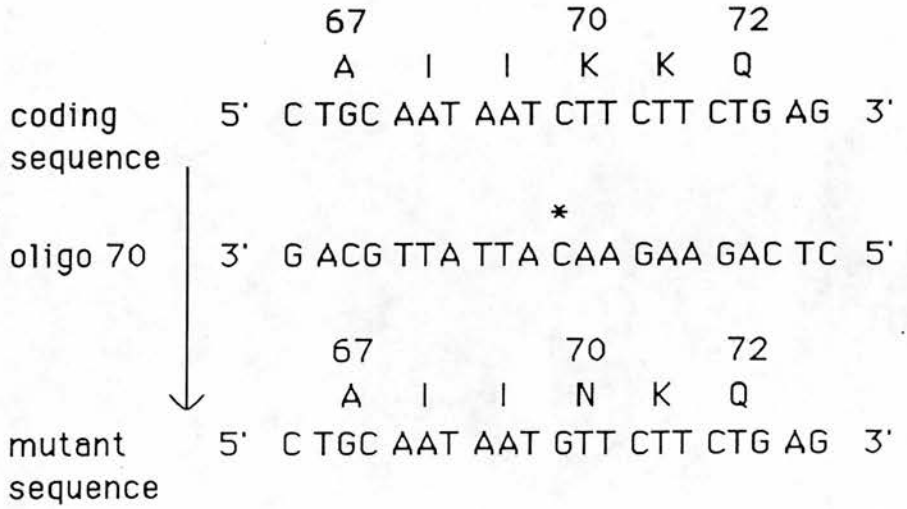


Figure 3.14C Oligonucleotide used to create mutant 70

Once the mutants had been made, the specific regions were checked by sequencing. The mutations generated are shown in figure 3.15. The inserts were removed by BamHI digestion and cloned into the expression vector pMA91. Recombinant plasmids were then mapped to ensure correct insertion. All mutant vectors were transformed into yeast strain AH22 and plated out on YOM LEU- plates. Transformants were then analysed by plasmid rescue and restriction mapping.

There appears to be some controversy whether yeast transformation is mutagenic. Having already sequenced and verified the presence of the desired mutations, it was decided to sequence the complete gene of each mutant as well as that of the wild type. In order to save having to subclone these genes back into bacteriophage M13 from the expression vector, a double stranded protocol was attempted. This method proved unsuitable in that runs of the same base were unreadable on sequencing gels. The inserts would have to be subcloned into M13. There was a problem in that the insert could not be excised using BamHI since the restriction site had been lost on cloning into the BglII site of the expression vector.

Plasmid pMABLG was mapped with a variety of restriction enzymes in order to find a site that would excise the gene in a small enough fragment to allow the use of the M13 universal primer to sequence the beginning of the BLG gene. No convenient sites were found. A large fragment would have to be subcloned in a particular orientation and a new oligonucleotide designed to sequence the beginning of the gene. When annealed, the 3' end of this oligonucleotide, called 050G (fig. 3.16), should lie 38 bases

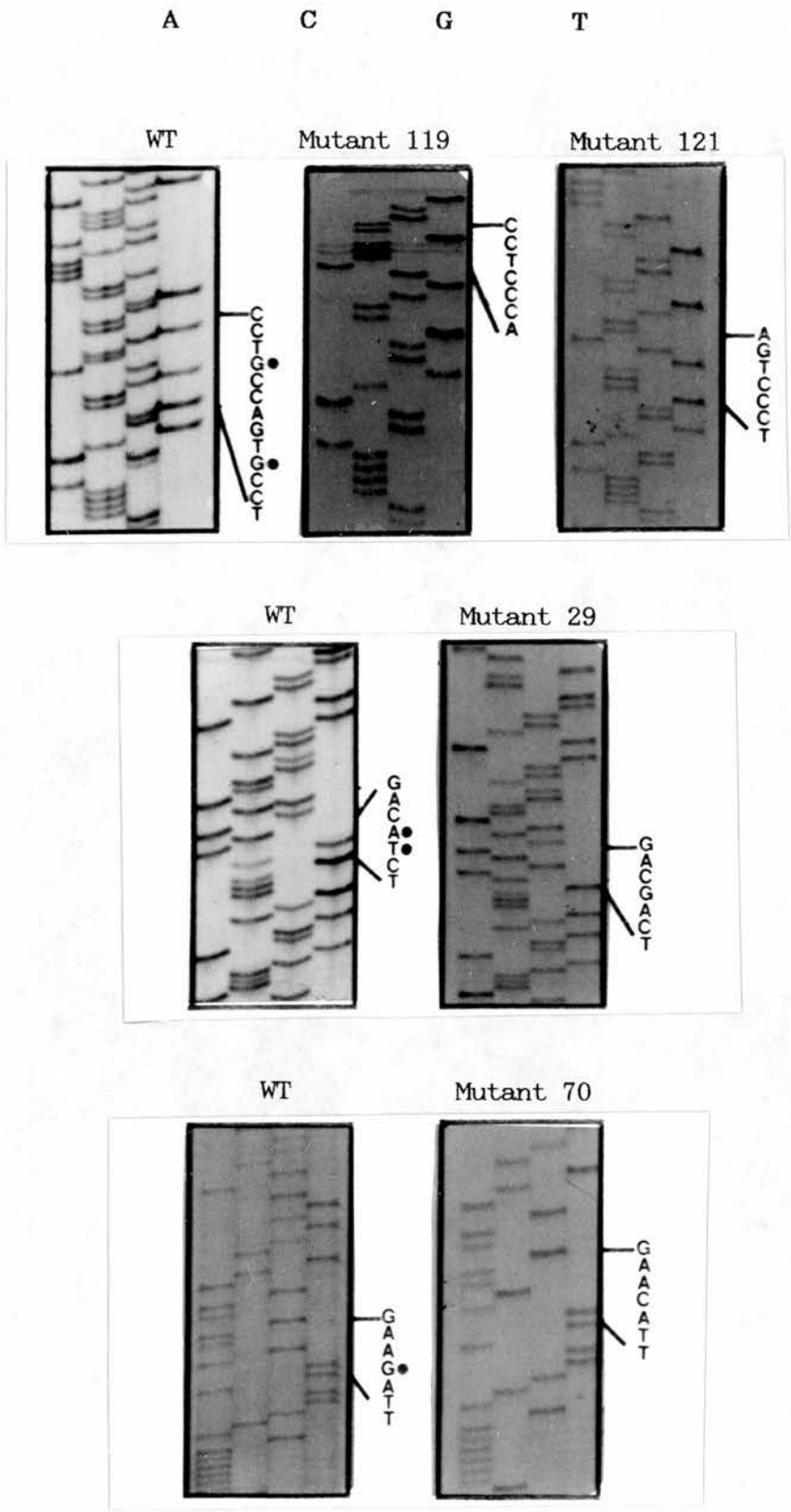


Figure 3.15 DNA sequence analysis of mutant BLG genes.

5' GGAGTAATTATCTACTTT 3'

Figure 3.16 Oligo 050G. This oligonucleotide was used as a primer to allow sequencing of the begining of the BLG gene.

Figure 3.17 DNA sequence comparison of oligonucleotide 70 and bacteriophage M13.

upstream from the ATG codon of the BLG gene. Mutant oligonucleotides 29 and 70 could also be used as primers to allow sequencing of the rest of the gene.

The 3kb SalI/EcoRI fragments, from all pMABLG vectors, containing the the wild type and all mutant BLG genes were cloned into M13. Clear plaques were analysed by the T tracking procedure to verify the presence of inserts. Oligonucleotide 70 was used as the primer and the M13 recombinant, MPBLG, as a positive control. All of the plaques and the control produced aberrant band profiles but since the bands had strong intensities, some sequencing artefact was suspected. However, when the plaques were sequenced using oligonucleotide 70 as a primer, it was discovered that all the plaques were non recombinants. It was found that oligonucleotide 70 had a strong recognition for a particular stretch of M13 DNA (fig. 3.17), which accounted for the aberrant T tracking profiles. The control profile was complicated by the primer initiating T tracking at two different sites. In order to avoid having to design a new oligonucleotide to replace oligonucleotide 70, another attempt was made at sequencing the genes without subcloning into M13.

All recombinant pMA91 derived plasmids were digested by SalI and EcoRI and the fragments separated by gel electrophoresis. Each 3kb SalI/EcoRI fragment containing a different BLG variant was purified by electroelution using the Biotrap and a different double

stranded sequencing protocol, described in the Materials and Method section, was followed. The three oligonucleotides, mentioned above, were used as primers.

The BLG gene was sequenced from a linear fragment, rather than from the complete expression vector for two reasons. Firstly, to minimise the risk of the oligonucleotides annealing to locations other than those desired, as much of the vector had not been sequenced. Secondly, purifying the fragment with the Biotrap was an excellent way of removing any contaminants, thus avoiding many sequencing artefacts.

The wild type and all four mutant genes were successfully sequenced using this method. No mutations, other than those synthesised, were detected in any of the genes. It could now be assumed that any alterations in protein behaviour resulted from mutations deliberately generated.

Now that all of the mutations had been made and the full coding sequence of each verified, it was necessary to determine whether the mutants would be successfully secreted.

3.8 Expresson of BLG mutants.

Small 10ml cultures of yeast transformed by all mutant and control recombinant and non-recombinant plasmids were grown in YOM LEU- medium. The cells were harvested, lysed and extracts analysed by SDS-PAGE. Medium from each culture was concentrated to 1ml by

ultrafiltration and then freeze dried. Precipitates were resuspended in 50ul of distilled water from which 10ul samples were analysed by SDS-PAGE (fig. 3.18). Both gels were stained with Coomassie Blue.

No BLG was detected in any of the cellular extracts. The gel of the medium samples showed that mutants 29, 70 and 121 were all secreted into the medium but there was no band corresponding to mutant 119. The search for intracellular BLG was repeated using Western analysis (fig. 3.19). All lanes, including the negative control, appeared to have the same doublet of bands associated with 2-mercaptoethanol-treated BLG. The Coommassie blue stained cellular extracts in figure 3.8A showed two yeast bands almost identical in size to BLG. The antiserum in the present experiment was affinity purified, however, results showed that it recognised many yeast cellular epitopes. Since the cellular yeast bands in question stained strongly with Coommassie blue, it was not unreasonable to expect such an antiserum to generate an equally strong signal. Even though all lanes from the Western gel contained a large amount of background noise, a band comparable in size to the larger band of the BLG control was clearly visible in the mutant 119 lane that was not present in any other. It seemed likely that this band represented unsecreted mutant 119 BLG. Conclusive evidence will only be obtained when a specific antiserum is developed or when the protein can be purified in sufficient quantities to sequence. There was no information regarding its location within the cell.

All mutant proteins that were secreted into the medium were not detected from intracellular crude extracts. The purported detection of intracellular mutant 119 protein by Western analysis suggests that the secretion pathway for this particular mutant has in some been inhibited post translationally and may result from problems of protein instability. The amount of mutant 119 detected within the cell did not match the secreted amounts of the other mutants i.e. there was no equivilent intracellular accumulation of mutant 119 protein. This may suggest that the protein is being degraded. Crude cell extracts can be prepared from exponentially growing cultures at various time points following addition of translational inhibitors such as cyclohexamide. Using an anti-BLG antiserum to detect mutant 119, loss of signal will occur if the protein is being degraded. If so, the site of degradation may be ascertained by the fractionation of whole cell lysates on Percoll gradients followed by SDS-PAGE and Western analysis. However, loss of detectable intracellular BLG may result from aggregation of the protein. Aggregates, formed by intra and inter chain disulphide bridges, would have been removed during the centrifugation step when preparing the soluble cell lysate fraction. Since BLG aggregates are difficult to solubilise with SDS and 2-mercaptoethanol alone, long incubations in 8M urea and reducing agents would be required. Any BLG solubilised by such a procedure could then be detected by Western analysis.

Figure 3.18 SDS-PAGE of native and mutant ovine BLGs secreted into the growth medium. Medium from each yeast culture (denoted above) was concentrated 20 fold and 10ul from each concentrate along with 8ug of a bovine BLG standard was separated on a 9% polyacrylamide gel. The gel was then stained with Coomassie Blue.

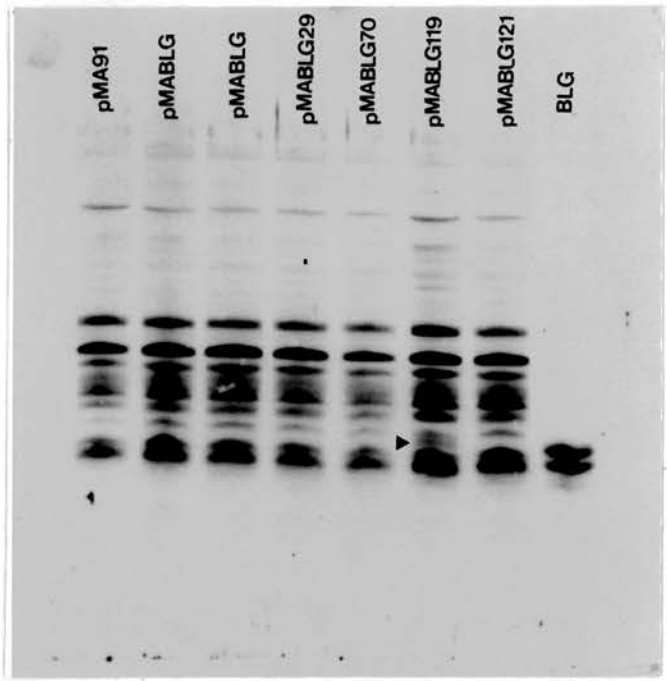


Figure 3.19 Western blot of cellular extracts. Samples (5ul) of crude cell extracts prepared from cultures carrying the plasmids denoted above, were separated on a 9% polyacrylamide gel. Following electroblotting onto nitrocellulose, the membrane was probed with a rabbit antiserum raised against bovine BLG. Bound antibodies were detected by a futher incubation with peroxidase conjugated anti-rabbit IgG followed by development using the Amersham ECL procedure.

▸ = position of mutant 119 BLG

Now that the expression of all mutants and secretion of three had been verified, it was necessary to concentrate on refining a large scale protein purification procedure which would hopefully be valid for the native protein and, ideally, all subsequent secreted mutants.

3.9 Small scale purification of BLG

Small test experiments were set up in an attempt to enhance the amount of BLG secreted and optimise protein purification procedures.

3.9.1 Secretion of BLG in different media

Cells containing the native ovine gene were grown in 5ml of YOM LEU- medium. Aliquots were then subcultured into three flasks containing 50ml of YOM LEU- medium and 50ml of YPD medium, at a density of $1x10^6$ cells per ml. The cultures were aerated at 30°C for 16, 24 and 48 hours. After the appropriate time had elapsed, the cells from a pair of cultures grown in YPD and YOM were harvested and the medium concentrated with an Amicon ultrafiltration cell. This medium was further concentrated to 400ul using Millipore Ultrafree-MC units which consisted of a small cup with a filter sealed at the bottom. This filter had a M_r cut off of 10,000. The cup was then inserted into a 1.5ml Eppendorf tube and spun at low speed in a microfuge. Medium passed through the filter and was collected in the Eppendorf tube leaving a small volume, containing molecules larger than 10kDa, in the cup. The

cup could then be refilled with more medium and the process repeated until the desired concentration was obtained. This latter procedure was not suitable for concentrating YPD medium because the filter became quickly blocked. An attempt was made to remove these blocking components by centrifugation but no pellet was obtained. This effect was also observed with YOM medium but to a much lesser extent, probably due to the simpler composition of this medium. Each 5ml YPD sample was instead freeze dried and resuspended in distilled water to a final volume of 400ul. 10ul from each of the above medium samples was boiled in sample buffer containing 2-mercaptoethanol and analysed by SDS-PAGE (fig. 3.20A).

The cells, for some reason, grew more slowly in YOM LEU- medium than expected so it is probably unwise to make any absolute comparisons. The gel was not particularly clear and BLG was detected only in the 48 hour lane from the YOM samples. Along with the BLG control, it migrated faster than the BLG from the YPD samples, which is difficult to see. This may be due to some retardation effect caused by components in YPD medium. One thing evident is that the BLG bands gradually disappeared in YPD samples. Loss of plasmid over a period of time under non selective conditions was expected, loss of protein was not. It is possible, that once secreted into YPD medium, BLG is subjected to time dependant denaturation and/or degradation or even conversion to higher molecular mass species. This degradation may be due to the release of proteases from dead cells, the amount of which will increase the longer cells are maintained at stationary phase. No similar conclusions can be drawn from YOM samples with this

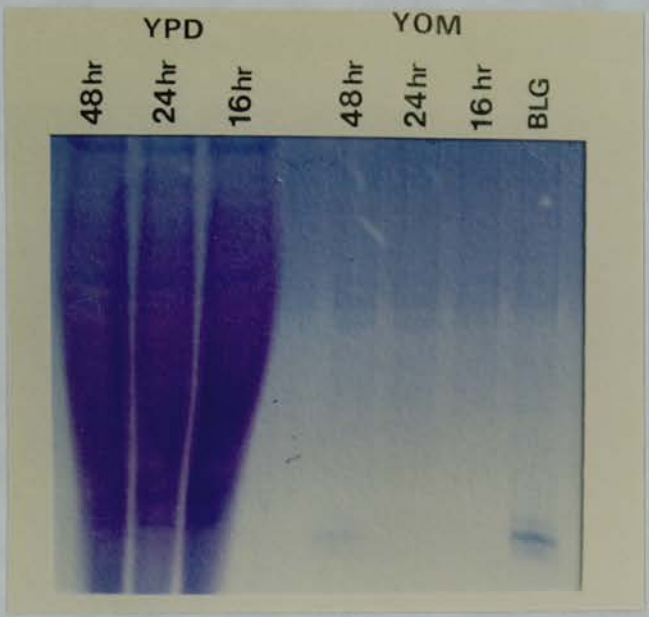


Figure 3.20A SDS-PAGE of BLG secreted from yeast grown in YPD and YOM. The boiling mix contained 2-mercaptoethanol.

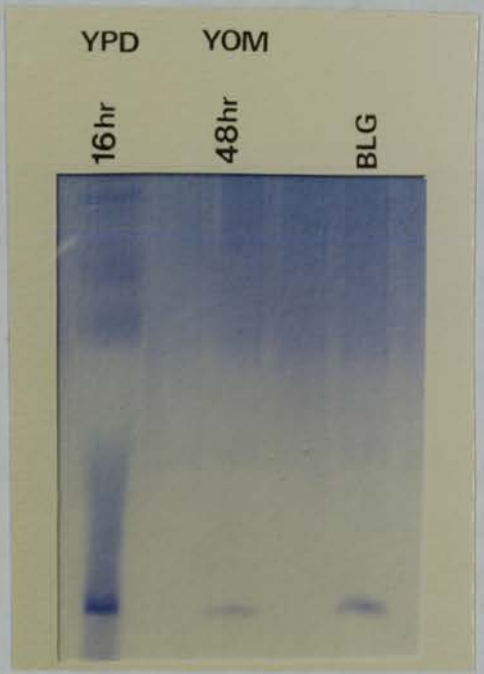


Figure 3.20B SDS-PAGE of dialysed medium samples. 2-mercaptoethanol was ommitted from the boiling mix.

particular experiment. It was difficult to make a direct comparison of yield of protein between cells grown in different media. As already stated, cells grew more slowly in YOM LEU- medium but should have reached a sufficiently high density after 48 hours to allow some comparable conclusions. BLG was difficult to visualise on the gel owing to the effect of 2-mercaptoethanol on an already small concentration of protein, and to the high background colouration in the YPD samples. The 16 hour YPD sample was dialysed against distilled water and this time 15ul samples of this and the 48 hour YOM sample was boiled in sample buffer without 2-mercaptoethanol and analysed by SDS-PAGE (fig. 3.20B).

BLG from both samples now ran at the same rate. Although the band width was smaller, there appeared to be a much greater amount of BLG present in the YPD lane compared to the YOM lane. This is probably due to the higher cell density of the culture rather than any increased efficiency of secretion.

It appears that YPD is a good growth medium for obtaining large amounts of BLG. The gradual loss of protein and the increased loss of plasmid copy number in cells grown in this medium may be offset by the overall higher yield obtained and the shorter length of time needed to grow cultures. It remains to be seen whether BLG will be easily purified from such a complex mixture.

3.9.2 Elution of BLG from DEAE cellulose by varying pH

A major problem associated with BLG purification involves its initial concentration from large volumes of medium. Ultrafiltration is time consuming but it may be possible to concentrate the protein by binding it to ion exchange beads which are gently stirred in the medium. The beads would then be allowed to settle and in turn transferred to a column for elution. This idea was tested on a small scale.

Bovine BLG was added to YPD medium at varying pH's to a concentration of 250ug per ml. Samples (4ml) of each medium batch were added to separate 5ml Falcon tubes containing 150mgs of DEAE cellulose. Tubes were gently agitated for one minute and the cellulose was gently centrifuged at 1000rpm for 20 seconds. Samples (1ml) were removed from each supernatant and freeze dried and the remaining supernatant discarded. The cellulose pellet was gently resuspended in 4ml of 20mM sodium phosphate buffer at the appropriate pH, mixed gently and centrifuged. This step was repeated using fresh buffer and 1ml aliquots from each washing step were freeze dried. The cellulose pellet was finally resuspended in 4ml of 20mM sodium phosphate buffer containing 200mM sodium chloride, still maintaining the appropriate pH, and treated in the same manner. All freeze dried samples were resuspended in 100ul of distilled water from which 5ul was analysed by SDS-PAGE without 2-mercaptoethanol (figs. 3.21A, B and C).

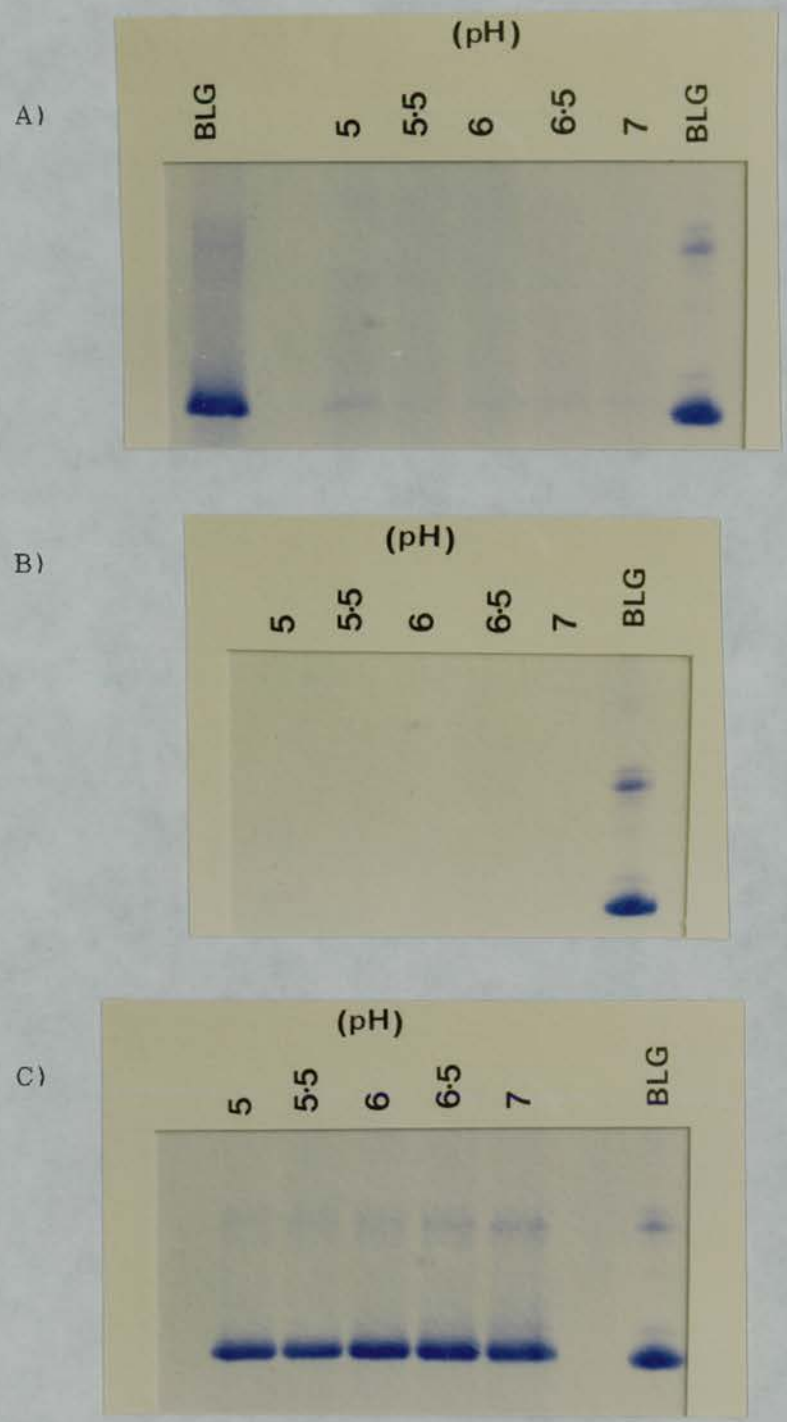


Figure 3.21 SDS-PAGE of bovine BLG eluted from DEAE cellulose by varying pH.

A) Amount of BLG remaining in the medium after binding to DEAE cellulose. Lane 1, concentration of BLG in the medium.

B) 20mM salt wash.

C) Step elution of BLG using 200mM salt.

There are two advantages in using DEAE cellulose as an ion exchange matrix. It shrinks very little when subjected to large variations in salt concentration and it has no, or very few, gel filtration properties. Figure 3.21A shows that BLG has efficiently bound to the cellulose at all pH's when compared to the total amount of protein present in the same volume (lane 1). Figure 3.21C shows that, by comparing the band intensities with lane 1 of figure 3.21A, the protein has been efficiently eluted by the 200mM sodium chloride step wash. BLG had previously been eluted from a DEAE Sephadex column in a gradient ranging from 0 to 100mM sodium chloride. It was felt that, in this experiment, a single step wash of 200mM sodium chloride should efficiently elute all BLG from the matrix and therefore serve as the upper limit required to elute the protein.

3.9.3 Elution of BLG from DEAE cellulose by varying salt concentration.

A very similar experiment to the one above was set up to study the elution of the protein at varying salt concentrations at a constant pH.

Bovine BLG was added to YPD medium, at pH 6.5, and transferred to Falcon tubes containing DEAE cellulose. Following mixing and centrifugation, 1ml aliquots were freeze dried and the remaining supernatant discarded. All pellets were washed twice in 20mM sodium phophate buffer, at pH 6.5. The cellulose pellets were then gentlly resuspended in 4ml of salt buffers of varying concentration

and 1ml aliquots of each were freeze dried. The pellets were finally subjected to a 200mM sodium chloride wash and aliquots were treated in the same manner. Samples from all the washes were then analysed by SDS-PAGE (figs. 3.22A and B).

Figure 3.22A once again showed the efficient binding of the protein to the cellulose when comparing the total amount of BLG present in the same volume (lane 8). Figure 3.22B, lanes 1 to 7, showed that BLG is poorly eluted at salt concentrations of 75mM and lower, and only eluted at reasonable levels at concentrations higher than 100mM. Correspondingly, the final wash at 200mM (lanes 8 to 14) showed the amount of protein still bound to the cellulose decreased in tubes that had previously been washed in consecutively higher concentrations of salt.

These two experiments served as a rough guide to the conditions required for the initial concentration and primary purification of BLG from larger scale cultures. There were, however, limitations to be kept in mind. The medium conditions used were not totally analagous to those of a growing culture and the protein would be present in the medium for a considerably longer period of time. Extra constituents either secreted from the cells or present as a result of cell death may have an effect on the protein outwith that of degradation. In this experiment, BLG eluted over a broad range of salt concentration. The mechanical force supplied by gentle agitation might have induced the separation of the protein from the matrix at a slightly lower salt concentration than would be expected when the protein is eluted from a column.

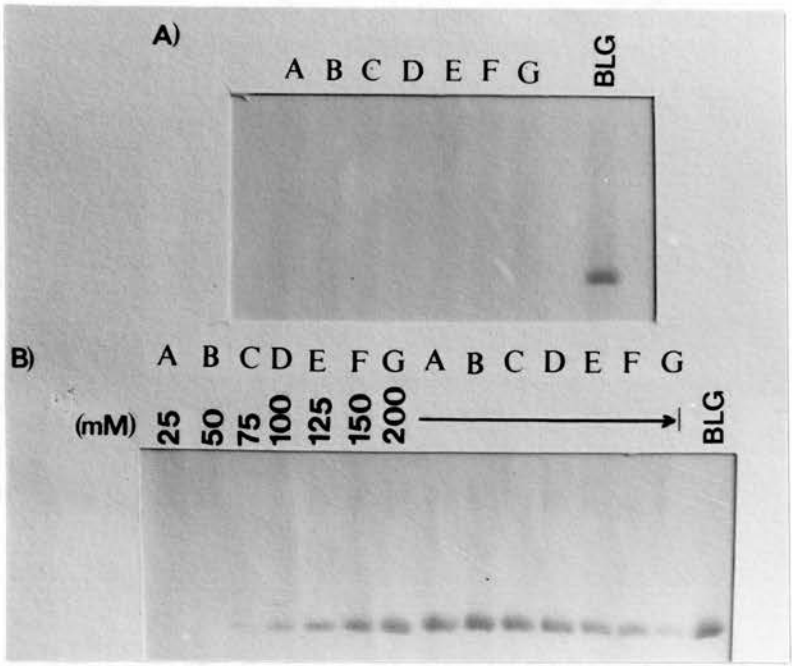


Figure 3.22 SDS-PAGE of bovine BLG eluted from DEAE cellulose, at pH 6.5, by varying salt concentration.

A) Binding of BLG to DEAE cellulose

A-G. Amount of unbound BLG remaining in the medium

BLG. Concentration of BLG in the medium

B) Elution of BLG at varying concentrations of salt		Step elution of BLG remaining after the first salt wash
A. 20mM	---------->	A. 200mM
B. 50mM	---------->	B. 200mM
C. 75mM	---------->	C. 200mM
D. 100mM	---------->	D. 200mM
E. 125mM	---------->	E. 200mM
F. 150mM	---------->	F. 200mM
G. 200mM	---------->	G. 200mM

These experiments have shown that the binding of BLG to ion exchange beads by gentle stirring is an efficient and rapid way of isolating the protein from large volumes of medium. The protein could either then be fractionated on a gradient, the upper limits of which have been determined, or eluted in a single step to allow further fractionation on another column.

3.10 Large scale purification of BLG from YPD medium.

Cells transformed with plasmid pMABLG were grown in four 100ml cultures containing YOM LEU- medium. Each of these was subcultured into 500ml of YPD and grown overnight. The cells were removed by centrifugation and the pH of the medium was adjusted from pH 5.8 to pH 6.5 with 1M sodium hydroxide. The medium was then added to two 500ml centrifuge bottles containing 50g of DEAE cellulose and gently agitated for two to three minutes. The beads were separated from the medium using a scintered glass funnel and then returned to the centrifuge bottles. The whole process was repeated until all the medium had been treated. The matrix was then washed in the scintered glass funnel with 20mM sodium phosphate buffer, pH 6.5, until the eluent ran clear. It was then transferred to a centrifuge bottle and sodium phosphate buffer added to form a thick slurry. This slurry was poured into a column to form a bed 5cm by 12cm. The cellulose was dark brown in colour showing it had bound many constituents other than BLG. The column was washed in 20 mM sodium phosphate buffer containing 200mM sodium chloride which resulted in the migration of a dark brown band down the column. Although the eluent was brown in colour, the column was also brown

suggesting that it still retained a lot of material. This procedure not only served as a concentration step but also allowed the separation of many contaminants. The eluent was dialysed in 20mM sodium phosphate buffer, pH 6.5, and loaded onto a column of fresh DEAE cellulose with a bed size of 2.2cm by 10cm. This column was subsequently judged to be larger than necessary since constituents in the loading eluent bound to the top 0.5cm of the column. The column was then washed with a 70mM sodium chloride step to remove contaminants at a salt concentration at which BLG should remain bound, and then eluted in a 70mM to 350mM sodium chloride gradient. This produced the elution profile in figure 3.23.

Material eluted by the 70mM sodium chloride wash was concentrated by ultrafiltration and samples from this and from eluent fractions from the gradient were analysed by SDS-PAGE. A small amount of BLG was detected from the initial wash. This was not unexpected since a small amount of protein was eluted in the small-scale experiment at a similar salt concentration. No BLG was detected from unconcentrated gradient fractions. It was assumed at this stage that the apparent lack of protein was a result of its dilute concentration. Material from the gradient eluted as a broad peak, the top of which was off the scale of the chart recorder. An attempt was made to increase resolution by using a smaller column and a broader gradient. All eluent fractions from the above gradient were pooled, concentrated and extensively dialysed in 20mM sodium chloride buffer, pH 6.5. The supernatant was then loaded onto a fresh DEAE cellulose column, 2.2cm by 2.5cm, and eluted with

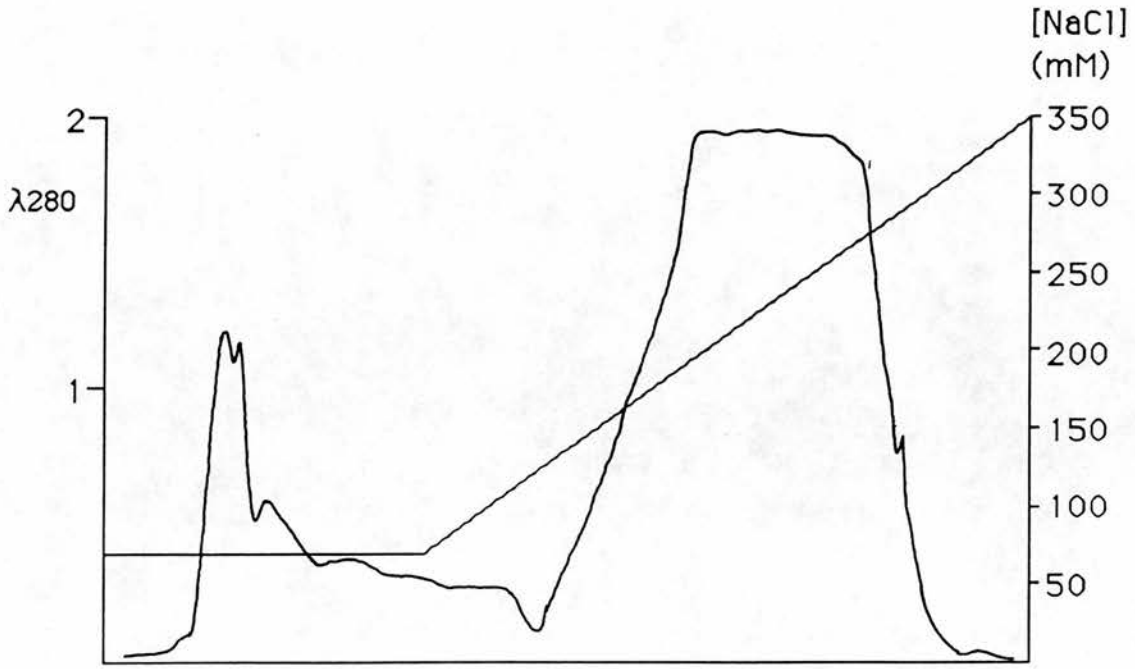


Figure3.23 Elution profile from DEAE cellulose (I)
Bed size: 2.2 x 10cm
Eluent: 70mM wash followed by a 70 to 350mM sodium chloride gradient in 20mM sodium phosphate buffer, pH 6.5

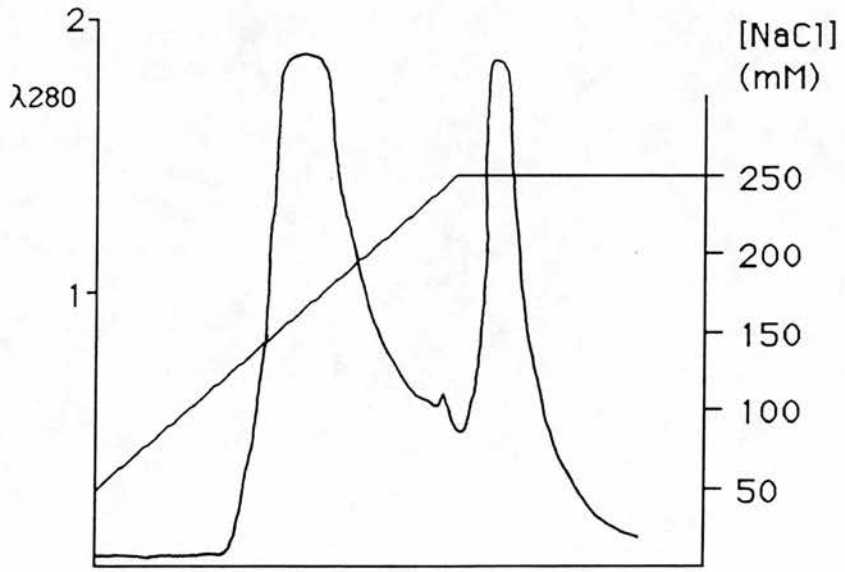


Figure 3.24 Elution profile from DEAE cellulose (II).
Bed size: 2.2 x 2.5cm.
Eluent: 50 to 250mM sodium chloride gradient in 20mM sodium phosphate buffer, pH 6.5, followed by a 250mM salt wash.

a 50mM to 250mM sodium chloride gradient followed by a 250mM step wash. The elution profile (fig. 3.24) showed that material eluted from the column in two major peaks. SDS-PAGE of fractions from the peak produced by the gradient showed very small amounts of protein of a size comparable with that of BLG. Higher molecular mass bands were present but they did not seem to correspond in size to higher molecular mass BLG aggregates seen in previous experiments. No bands corresponding to the size of BLG were detected from the step wash.

The yield of protein from the columns was only a tiny fraction of the expected amount. The cells were obviously synthesising BLG and it was safe to assume that the protein had been bound by the ion exchange beads. It was not clear what happened to the protein once on the column. The small-scale experiments showed that BLG was efficiently eluted in salt concentrations of 200mM. Therefore, it was assumed that the initial 200mM salt wash had eluted the protein along with a large amount of contaminants. Subsequent loading onto a fresh column and elution with 70mm salt revealed the presence of a small amount of BLG. This may have been a small subpopulation, the rest of which may have been in varying degrees of aggregation or degradation. It was possible that prolonged association with contaminants on the column, known to have highly "sticky" properties, or concentration by ultrafiltration with such contaminants may have resulted in the formation of complexes, from which the protein may not have been readily eluted. The columns were not washed with high salt buffers to elute the protein. Even

if this procedure had proved successful in eluting the protein, it was decided that YPD medium was too complex to allow simple purification of BLG.

3.11 Purification of BLG from YOM medium.

On this occasion a slightly different approach was chosen in which the medium was pumped directly onto an ion exchange column.

A 3 litre culture was grown in YOM LEU- medium to stationary phase. The cells were removed and the pH of the medium adjusted from 3.1 to 6.5. The medium was then pumped at 4°C directly onto a DEAE cellulose column, 2.5cm by 5cm. Material absorbed on to the top 1cm of the column, judged by the slight yellow colour. The column was then washed with a 250mM sodium chloride step and the eluent was analysed by SDS-PAGE. Only a very small amount of BLG was detected.

Once again, the protein failed to elute from the column in significant amounts. YOM is much simpler in composition than YPD and it was hoped that the factors responsible for the loss of protein during isolation from YPD would either be eliminated or at the very least be greatly reduced. It was also possible that the elution problems were unrelated and that purification from two different media produced different sets of problems. It was unknown whether the protein existed in free association and only formed complexes, not readily eluted from columns, when brought into close proximity with high concentrations of medium

constituents or whether such complexes formed readily even when very dilute. Concentration by ultrafiltraion and by ion exchange beads both had the effect of bringing BLG into close association with medium constituents. Accumulation of such complexes on a column to form even larger and more complicated aggregates may not allow the protein to elute, at least at lower salt concentrations. This may not be true for such precipitates remaining in a supernatant. The very first large scale purification (page 83) involving ultrafiltration followed by ion exchange chromatography included an intermediate dialysis step in a weak salt buffer at a higher pH. Subsequent elution from the column was easily accomplished and at a salt concentration much lower than the steps used above. The release of protein from complexes may require lengthy dialysis incubations in buffers at the appropriate salt concentration and pH. The precipitates on a column may have complexed to such an extent as to have gone beyond the ability of such an incubation to solubilise the protein. It was also possible that the dialysis step had removed at least some of the low molecular mass contaminants causing aggregation.

At the present stage, initial concentration of BLG from medium by ion exchange methods had to be given up in favour of ultrafiltration. This latter procedure also had the advantage of allowing the protein to be detected before further purification.

3.12 Purification of BLG by f.p.l.c.

A 1 litre culture of pMABLG-transformed cells was grown in YOM LEU- medium. The cells were removed and the medium concentrated to 10ml by ultrafiltration. A precipitate formed which was removed by centrifugation and resuspended in 20mM sodium phosphate buffer, pH 6.5. The supernatant was dialysed against the same buffer. Samples of the supernatant, before and after dialysis, and the precipitate were analysed by SDS-PAGE (fig. 3.25). There was no difference between supernatant samples before or after dialysis, however, a considerable amount, probably greater than 50%, of the BLG secreted into the medium was present in the precipitate.

The supernatant was concentrated from 10ml to 200ul during which a small amount of precipitate was detected. The precipitate was removed and the salt concentration of the supernatant altered to 150mM with sodium chloride. The sample was then fractionated on a Superose 12 f.p.l.c. column and produced the elution profile in figure 3.26.

SDS-PAGE showed that the end peak contained BLG of the correct molecular mass (fig. 3.25, lane 6). It was puzzling to see four major peaks in the elution profile resulting from the fractionation of the dialysed supernatant sample that contained only two major bands especially since the BLG peak was by no means the largest. SDS-PAGE of the concentrated sample (fig. 3.25, lane 5) loaded onto the column showed the presence of two extra bands not present in the dialysed supernatant. This may leave one to conclude that

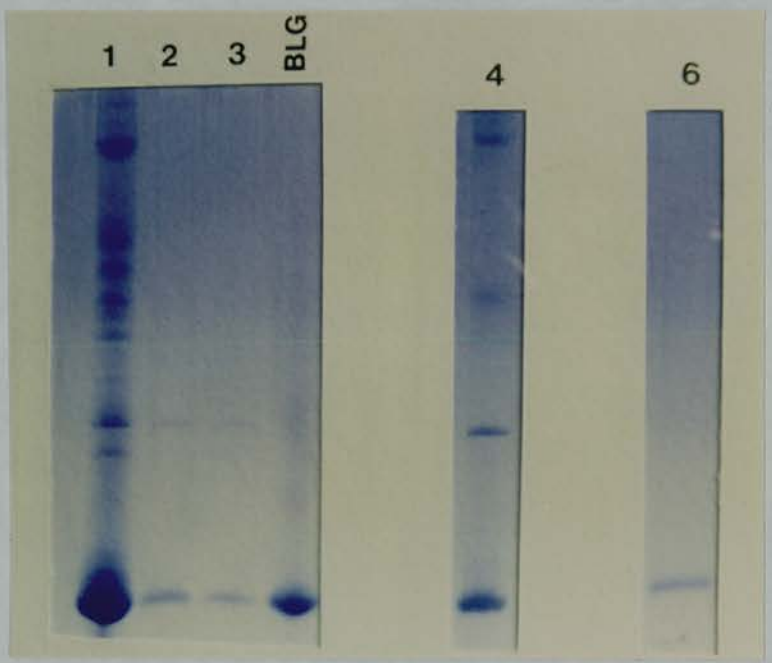


Figure 3.25 SDS-PAGE of medium concentrated by ultrafiltration.

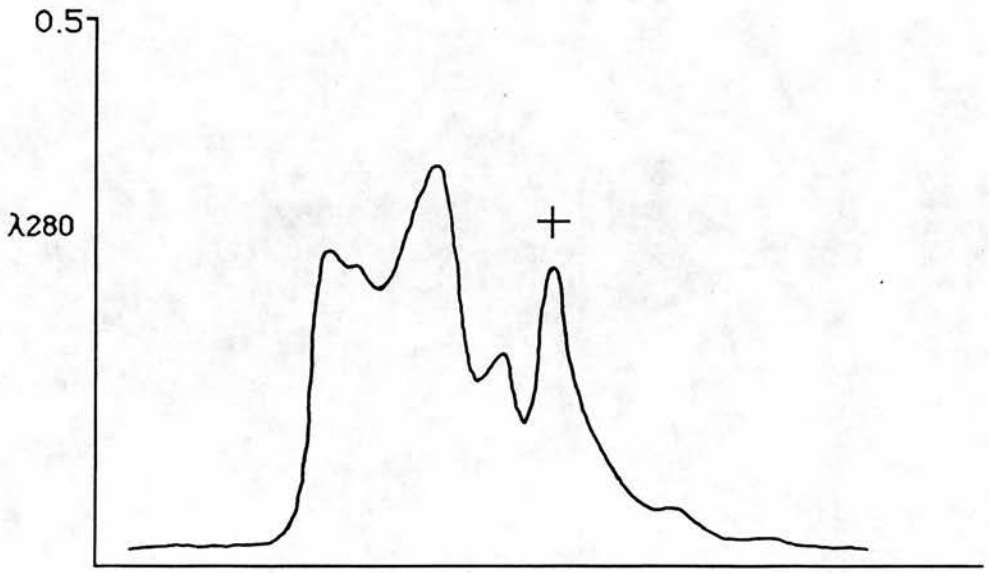
1) Precipitate resuspended and boiled in sample buffer.

2) BLG remaining in the supernatant prior to dialysis.

3) BLG remaining in the supernatant after dialysis.

4) Sample loaded onto the f.p.l.c. column. This sample consisted of a 50 fold concentration of the supernatant shown in lane 3.

6) Sample from the end peak of the f.p.l.c. elution profile shown in figure 3.26.



+ shows the peak corresponding to BLG as determined by SDS-PAGE

Figure 3.26 F.p.l.c. elution profile of concentrated medium containing BLG.

these extra bands represented aggregations of BLG and not concentrated higher molecular mass contaminants since no such bands existed in the dialysed supernatant of comparable intensity.

Although pure BLG was obtained at the end of the process, the yield was very poor. Conditions would have to be altered to solubilise the maximum amount of protein from the precipitate.

3.13 Altering concentrating conditions.

A 1 litre culture of cells transformed by pMABLG29 (mutant 29) was grown and the medium concentrated to 11ml. A precipitate formed during ultrafiltration. Three 1ml aliquots, containing suspended precipitate, were removed and dialysed under differing conditions. One was dialysed against 20mM sodium phosphate buffer, pH 6.5, at room temperature, another was dialysed against growth medium, pH 3, containing 350mM sodium chloride, at 4°C, and the last was dialysed in the same conditions as the second but at room temperature. After dialysis, precipitates were removed by centrifugation and resuspended in boiling mix. Precipitate from the remaining 8ml was removed and resuspended in boiling mix. The supernatant was dialysed against the phosphate buffer. Treatment of the 8ml fraction in this manner showed the amount of soluble BLG remaining in the supernatant after ultrafiltration and could be used for a comparison with the 1ml aliquots. After dialysis, equal size aliquots from all supernatant samples along with equivilent amounts of precipitate per volume of supernatant were analysed by SDS-PAGE (fig. 3.27).

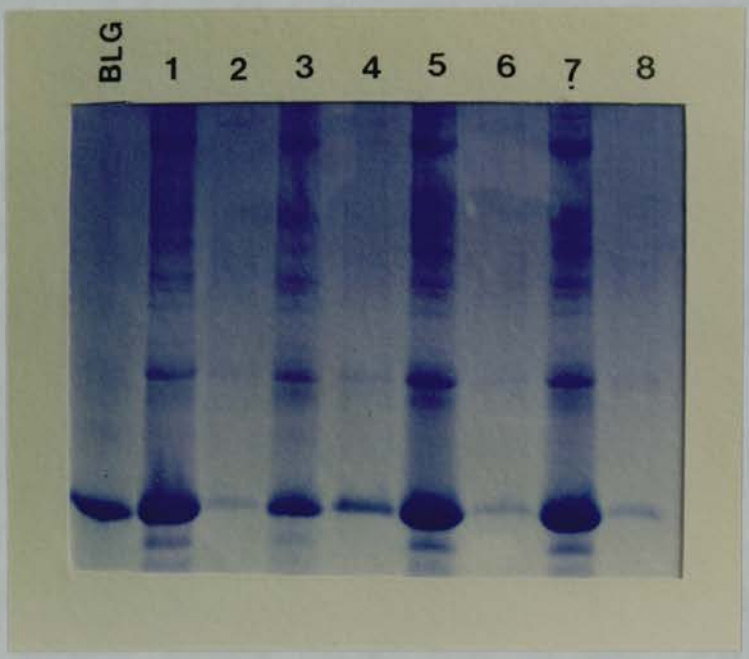


Figure 3.27 SDS-PAGE of BLG dialysed under varying conditions.

BLG

1. Precipitate, from the 8ml fraction.
2. Supernatant, from the 8ml fraction, dialysed against 20mM sodium phosphate, pH 6.5, at RT.
3. Precipitate remaining after dialysis against 20mM sodium phosphate, pH 6.5, at RT.
4. Supernatant fraction after dialysis against 20mM sodium phosphate, pH 6.5, at RT.
5. Precipitate remaining after dialysis against medium containing 350mM sodium chloride, pH 3, at 4°C.
6. Supernatant fraction after dialysis against medium containing 350mM sodium chloride, pH 3, at 4°C.
7. Precipitate remaining after dialysis against medium containing 350mM sodium chloride, pH 3, at RT.
8. Supernatant fraction after dialysis against medium containing 350mM sodium chloride, pH 3, at RT.

Dialysis at higher pH was the only procedure that solubilised the protein to any great extent. No extra protein was solubilised in medium samples containing high salt concentrations. Therefore, it did not seem likely that addition of salt to medium prior to concentration would inhibit the formation of precipitates.

All precipitate and supernatant samples were pooled and dialysed in 50mM sodium phosphate buffer, pH 7, containing 150mM sodium chloride, at room temperature. The precipitate formed by native ovine BLG was also dialysed as well as concentrated medium from a culture of pMABLG121 (mutant 121) transformed yeast. All three samples were subsequently fractionated on a Superose 12 f.p.l.c. column and produced the elution profiles shown in figures 3.28A, B and C. It was interesting to note that the shape of the profiles was variable. Any effect on the profile by medium constituents should remain reasonably constant, since the cells were all grown in the same medium. Differing peak sizes may reflect variations in abundance of certain sizes of BLG aggregates since only the peak intensities were variable, not their retention times. SDS-PAGE showed that the last major peak from each profile corresponded to BLG of the correct size. The desired peaks from each sample were pooled and refractionation showed that BLG could be purified to homogeneity (fig. 3.29).

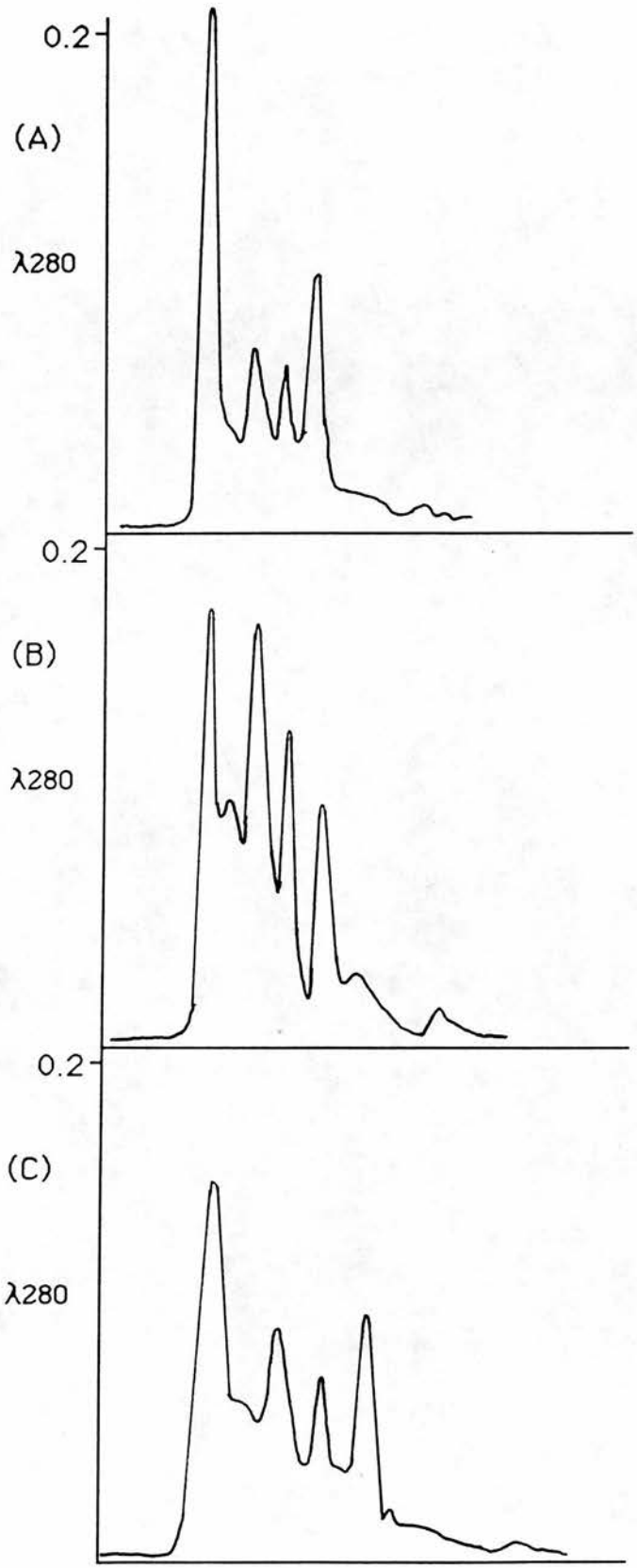


Figure 3.28 F.p.l.c. elution profiles of A) dialysed native ovine BLG precipitate, B) mutant 121, C) mutant 29.
Eluent: 150mM sodium chloride in 50mM sodium phosphate buffer, pH 7.

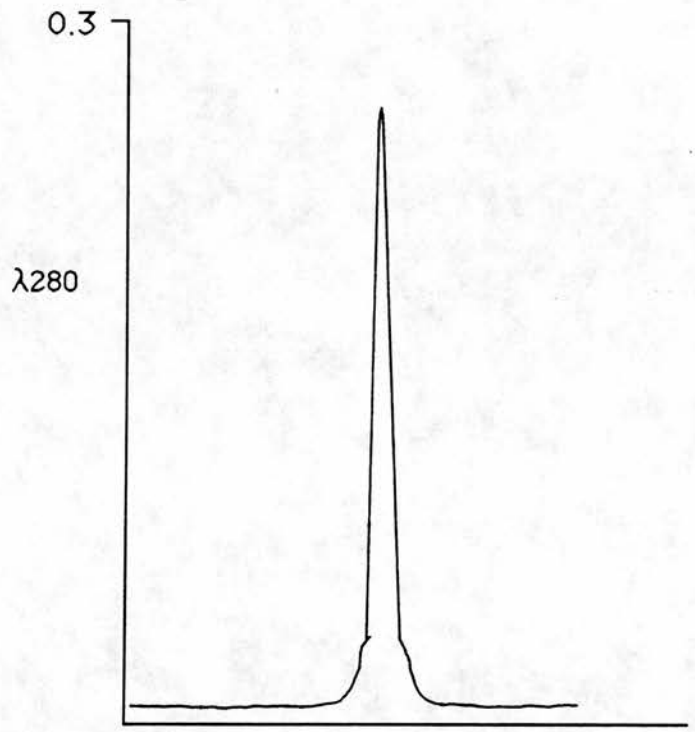


Figure 3.29 F.p.l.c. elution profile of pure BLG. This figure represents pooled fractions of the peak corresponding to BLG from the previous fractionation of concentrated medium. Subsequent reanalysis by f.p.l.c. showed the protein to be pure.

3.14. Characterisation of BLG proteins.

3.14.1 Native PAGE of BLG

Concentrates of native BLG and mutants 29, 70 and 121 from samples of media were analysed by native PAGE alongside bovine BLG (fig. 3.30A and B). The bovine control was composed of two variants, A and B. This reflected their differing mobilities under non denaturing conditions. All three mutants migrated at the expected rate with respect to the native ovine protein. Mutant 29 substituted an uncharged residue with a negatively charged one resulting in the net gain of a negative charge. Mutant 70 substituted a positively charged residue with an uncharged one. This substitution also resulted in the net gain of a negative charge. As a result, both mutants migrated together ahead of the native protein. Mutant 121 substituted one uncharged residue with another resulting in no overall change to the net charge. Therefore, it migrated alongside the native ovine protein.

3.14.2 Determination of molecular mass by f.p.l.c.

A Superose 12 column was individually calibrated at pH 7 with proteins of known molecular mass and a graph of molecular mass against retention time was plotted (fig. 3.31). The native ovine protein and all secreted mutants were run on the column and the retention times accurately noted. Using the equation derived from the calibration curve, all ovine proteins were found to have a M_r of 23,000. Monomers and dimers of BLG should have an approximate

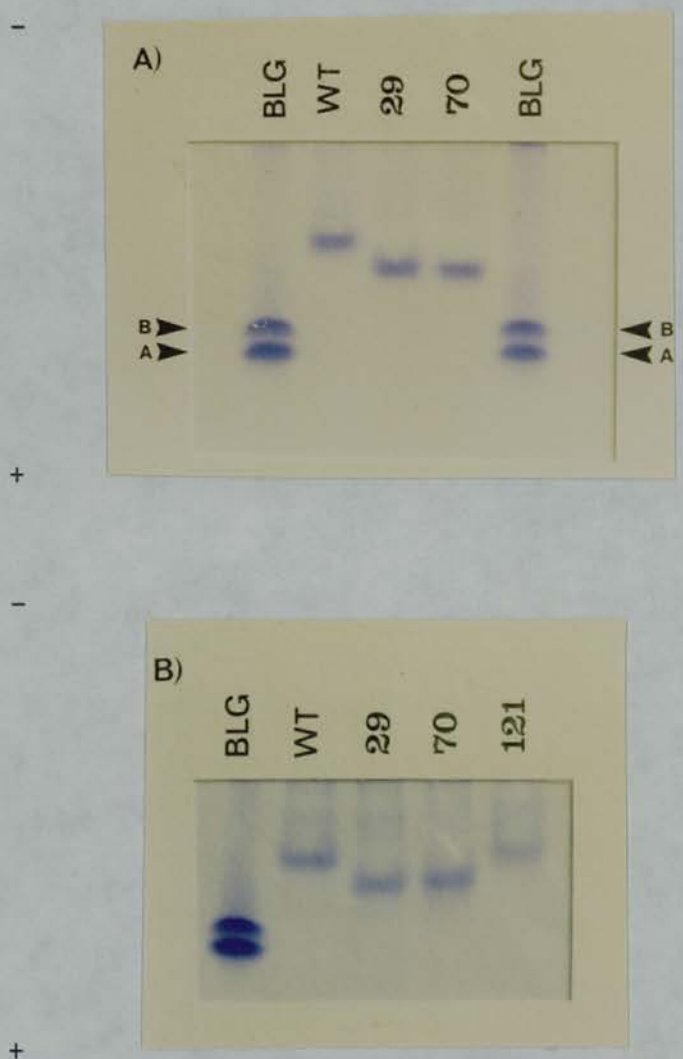


Figure 3.30 Native PAGE of BLG

A) 1,5 bovine BLG, mixture of A and B variants

2 native ovine BLG

3 mutant 29

4 mutant 70

B) 1 bovine BLG

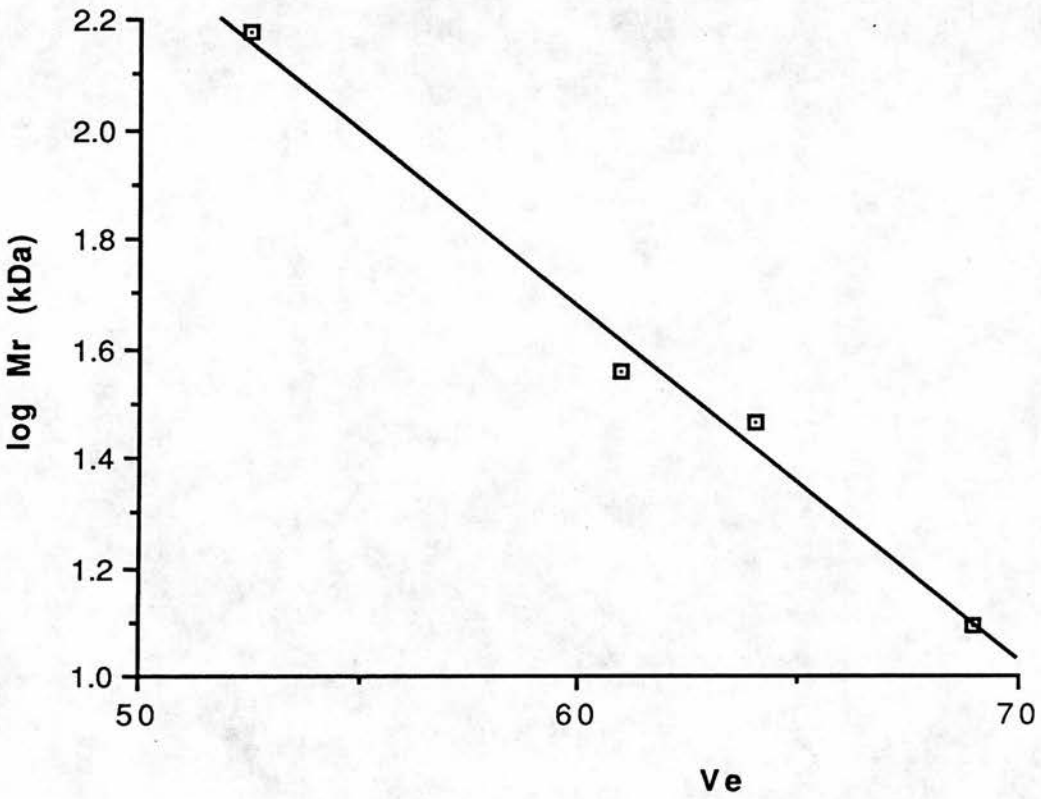
2 native ovine BLG

3 mutant 29

4 mutant 70

5 mutant 121

Figure 3.31 Superose 12 calibration curve.



alcohol dehydrogenase	150kDa
bovine BLG	36kDa
carbonic anhydrase	29kDa
cytochrome C	12.4kDa

Ve for all ovine BLGs = 65

M_r of 18,000 and 36,000 respectively. It is well known that bovine BLG exists as a dimer at physiological pH and there is evidence that the ovine form also does [165]. The evidence here suggested that all yeast secreted ovine proteins existed as monomers. Although the experimental M_r was 23,000 this may result from an inefficient separation of small proteins by the Superose 12 column. Since there was no difference in mobility between yeast secreted BLG and the bovine standard obtained from Sigma in denaturing gels, it is possible that the increase in M_r results from some noncovalent association with small medium derived components which detach during the boiling step prior to SDS-PAGE. If this value is correct, it is unclear why the ovine proteins, apart from mutant 29, should exist as monomers. It is possible that the proteins have a subtly different conformation from the true protein however, it is more likely that the protein may never be sufficiently concentrated to allow dimerisation to occur [166]. There is also the possiblity that the protein has been modified in some undetermined way thus inhibiting dimerisation or even affecting the structure as a whole. One possible modification is N-glycosylation. Bovine BLG has a single Asn-X-Thr glycosylation site at Asn152. However, it has been shown that this site cannot be glycosylated when X consists of a proline [167] and is supported by the fact that BLG is not glycosylated *in vivo*. Ovine BLG has the same site and SDS-PAGE has shown no difference in mobility between the two proteins. One can conclude that the protein secreted by yeast is not glycosylated. Studies on Cys mutant human lysozymes secreted by yeast [168] showed the presence of mixed disulphide derivatives between Cys95 and glutathione which affected

enzyme activity. There is no suggestion that this has occurred in secreted ovine proteins and is only mentioned as an example of an unexpected spurious modification.

During these experiments, it was noticed that when fractions containing the BLG peak were pooled and concentrated by ultrafiltration, a precipitate formed. In future, another type of ultrafiltration device may have to be used that induces less denaturation of the protein. Ammonium sulphate precipitation is another alternative but at the protein concentrations currently available, recovery of protein by such means would be very inefficient. The use of small ion exchange columns cannot be over looked. Pure protein could be eluted from such a column in a single step in a volume small enough to be useful.

3.14.3 Denaturation of ovine BLG in urea solutions at pH 2.8

Although the yield of protein recovered after f.p.l.c. fractionation was low, it was possible to study the unfolding of the protein in differing concentrations of urea and thus make a comparison of its stability with bovine BLG. An estimation of denaturation in increasing urea concentrations for bovine BLG was obtained using c.d. spectroscopy. The value at 225nm obtained when no urea was present was taken to represent the 100% native state and hence the fully folded conformation of the protein. Subsequent values were plotted as a percentage of folding against urea concentration (fig. 3.32).

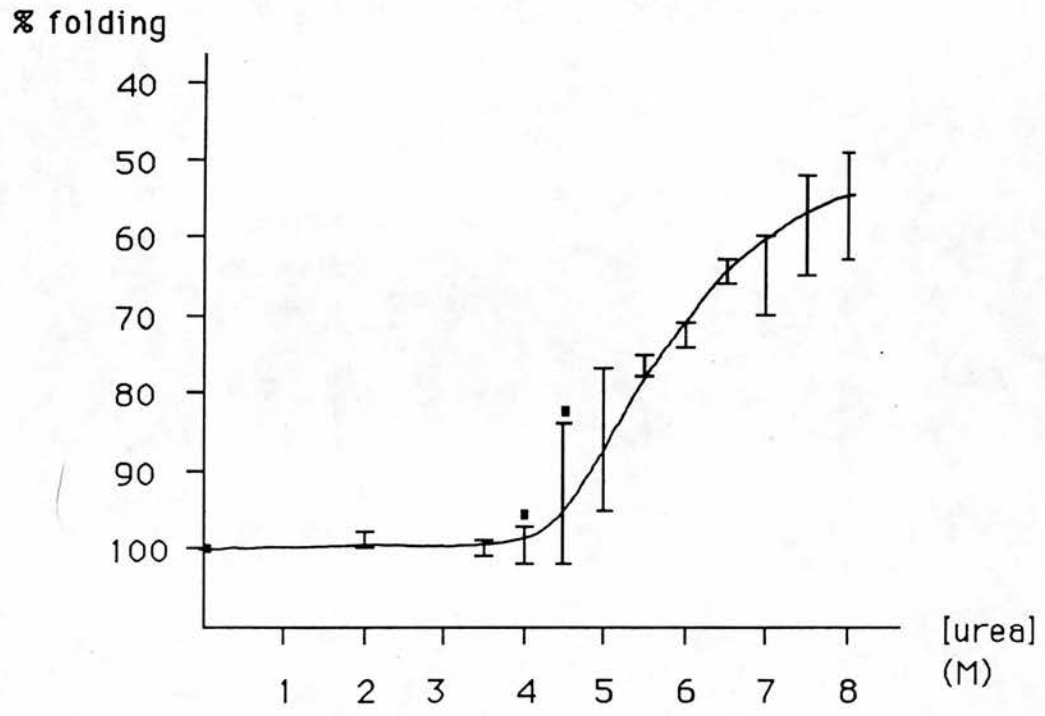


Figure 3.32 Unfolding of bovine BLG in urea. 100ug samples of BLG in 150mM potassium iodide, pH 2.83, were incubated in various concentrations of urea for 15 min at 20°C prior to c.d. analysis. Each urea concentration was repeated three times and gave the error bars above.

▪ = ovine BLG samples

There was only enough "native" ovine protein to measure three values. Although the points lay outside the error bars obtained for the bovine protein, little, if anything, could be concluded from the limited amount of information derived. The detector was working at extreme sensitivities and so any error may have been distorted. The protein may not have existed in its truly native form. Apart from any distortions in structure provided by its synthesis in yeast, concentration after f.p.l.c. fractionation in the previous step produced a precipitate. Therefore, protein remaining in solution may have already started down the road to denaturation before addition of urea. Even if sufficient values had been obtained, a true indication of its native structure would only have been elucidated by comparison to an ovine standard.

4.0 General discussion

A successful expression system for the synthesis and secretion of ovine BLG was developed using plasmid pMA91 which contained the PGK transcriptional control sequences, and the yeast strain AH22. Earlier attempts using plasmids with the GAL1 and ADH1 transcriptional control sequences were unsuccessful. During construction of these plasmids, the amount of noncoding DNA between the promoters and the initiation of translation signal was kept to a minimum since studies elswhere showed that the level of mouse liver cytochrome P-450 P1 expression in yeast increased as the amount of upstream noncoding sequences of the original cDNA was reduced [169]. The GAL1-BLG constructs contained no downstream transcription termination signals. This would have resulted in the synthesis of a larger mRNA that was probably not polyadenylated. The processes controling mRNA stability are not clear at present. There are cases where mRNAs lacking their poly(A)+ tails are subject to degradation, presumably by exonucleases, and other cases where it has been shown that larger mRNAs appear to be more unstable [170]. However, studies have been carried out on the expression of calf prochymosin in yeast using expression systems with and without transcription termination signals [171]. The plasmid without termination signals produced transcripts as large as 3.8kb whereas the plasmid containing termination signals produced a distinct transcript of around 1.75kb. Both expression systems produced equal amounts of activatable prochymosin. It is not clear whether the plasmids, containing the GAL1 promoter, produced viable mRNA. However, problems of aberrant transcripts

should not have arisen from the ADH-BLG construct which contained the transcription termination signals for the yeast ADH1 gene. The gene to be expressed from this ADH expression cassette requires the presence of an ATG start codon and a translational termination codon [159]. Both of these codons were supplied in the BLG insert which should have resulted in the synthesis of a viable polyadenylated transcript.

The problem may have been one of protein stability. All the BLG proteins synthesised by plasmids with the GAL1 and the ADH1 promoters had a tetra-peptide joined onto the front of the leader sequence. During the early stages of translation, yeast may not have recognised the N-terminal portion of the polypeptide emerging from the ribosome as a signal peptide and may not have directed the mRNA-ribosome complex to the rough endoplasmic reticulum. Although there is no hard evidence regarding the presence of the signal recognition particle observed in mammalian cells [172], yeast must rely on similar machinery to specifically direct secretory proteins to the rough endoplasmic reticulum. The formation of disulphide bridges only occurs within the endoplasmic reticulum and is catalysed by protein disulphide isomerase [173]. If translation had proceeded in the cytoplasm, the signal peptide would not have been excised and the correct disulphide bridges would not have formed. The protein would not have folded properly under these conditions and may have formed aggregates covalently linked by disulphide bonds and/or it may have been degraded by proteolytic enzymes. This scenario may seem plausible in light of the expression of mutant 119. The lack of secretion of mutant 119

suggests that it had also folded incorrectly and yet it was detected in cellular extracts by Western analysis. The difference may lie with the fact that this BLG had a functional signal peptide and would have been translocated into the endoplasmic reticulum. However, the amount of mutant 119 detected was very small and not what might be expected from the intracellular accumulation of a protein normally secreted. Eukaryote cells possess polypeptide chain binding proteins (PCBs), otherwise known as molecular chaperones, that are thought to be involved in the folding of nascent proteins and the unfolding of others prior to translocation through membranes [174]. Binding of these PCBs to nascent polypeptides is thought to limit the amount of nonspecific aggregation that would otherwise occur. Programmed stepwise release of these PCBs may allow proteins to fold correctly. Several members of this family are induced during periods of cell stress, eg. the hsp70 family, which may protect denatured proteins from degradation or help them refold. It is possible that the lack of secretion of mutant 119 results from interactions with PCBs. Since it is not beneficial to the cell to accumulate wrongly folded proteins in the endoplasmic reticulum, it is possible that mutant 119 was targeted for degradation. Studies involving the degradation of newly synthesised T cell receptor subunits identified two distinct degradative pathways, one lysosomal, the other pre-Golgi which may either be part of, or closely associated with, the endoplasmic reticulum [175]. The yeast cell vacuole provides a similar role to the lysosome of higher eukaryotes and is possibly the main site of protein degradation in yeast. There is no information regarding an alternative endoplasmic reticulum

related pathway in yeast. Even in the presence of these PCBs it is also possible that prolonged accumulations induce the formation of insoluble aggregates. Such insoluble aggregations were observed when human lysozyme was expressed in yeast [176].

It is not clear at present how the substitution of Cys119 with serine affects the tertiary structure of BLG. One obvious feature is the loss of the disulphide bridge with Cys106. This fact alone may be responsible for the altered stability of the protein. However, during folding, protein disulphide isomerase may catalyse the formation of a disulphide bond between Cys106 and 121. It is interesting to speculate that the formation of a disulphide bridge between these two cysteine residues may be the cause of unstable structural alterations. X-ray crystallography analysis has shown that the disulphide bridge always forms between Cys106 and Cys119. Formation of the alternative bond between Cys106 and Cys121 would require a 10Å shift between ß-strands G and H (fig. 4.0) which in turn may cause regions of instability.

Since protein mobility in non reducing and reducing polyacrylamide gels is affected by charge and conformation, it might be possible to reveal structural alterations between the two Cysteine mutants and the wild type not observed in SDS-PAGE. The migration of mutant 119 would have to be detected by Western analysis due to the contamination of cellular proteins. This experiment would not reveal the number of disulphide bridges within each mutant. Purification of mutant 119 by chromatographic means may prove extremely difficult, especially if the protein is structurally

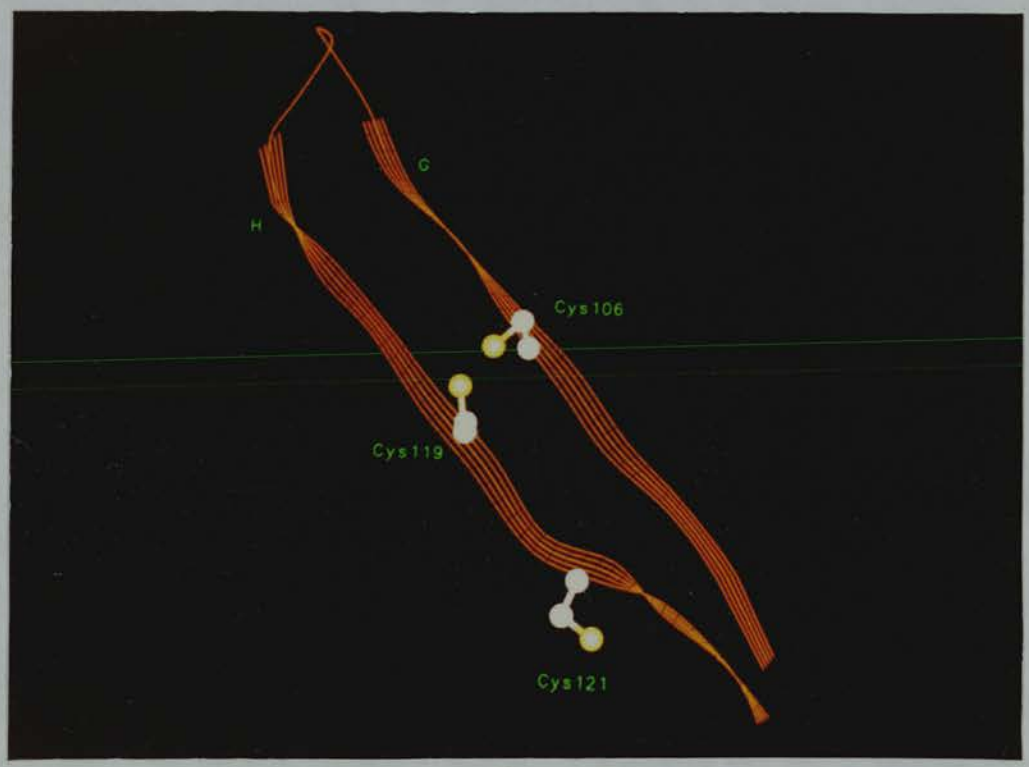


Figure 4.0 Position of the disulphide bridge between G and H ß-strands.

unstable. One possible way of purifying sufficient amounts for further analysis may rely on electroelution of the BLG band from a polyacrylamide gel. It may then be possible to radiolabel free sulphydryls in reducing and non reducing conditions with iodo[1-^{14}C]acetamide. Subsequent peptide digestion followed by 2D gel electrophoresis should show a variation in the pattern obtained under reducing and non reducing conditions. Any variations observed in the mutant peptide patterns will be detected by comparison with the pattern obtained from digestion of the wild type protein. Under reducing conditions, mutants 119 and 121 should both show four radiolabelled peptides and the wild type five. Under non reducing conditions, mutant 119 may show two (or more) radiolabelled peptides, whereas, wild type should have only one and mutant 121 should not have any.

However, apart from the possibilities discussed above, the problem may have been one of detection. Even though all the earlier expression studies were performed with small cultures, BLG was detected by SDS-PAGE from similar size cultures using the PGK expression system. The GAL1 and ADH1 promoters were known to be functional since these promoters, obtained from the same source, were used to express Drosophila ADH [160]. If BLG had formed insoluble aggregates, such aggregates would associate with the heavy membrane fraction following cell lysis and centrifugation. As a result, no BLG would be detected in the soluble fraction by SDS-PAGE.

Further research regarding protein purification is required to increase the yields of BLG. Even though initial yields were disappointing, it is worth perservering in order to answer important structural questions discussed in this thesis. Yields may be increased by using different yeast strains eg. a strain that carries the *pmr1* mutation can increase the efficiency of secretion of heterologous proteins [179]. Future manipulation of the coding sequence may also be facilitated by sucloning the PGK-BLG expression unit into a phagemid like pVT102u. This would enable expression studies and site-directed mutagenesis to be carried out in the same plasmid without further subcloning procedures. However, if yields of engineered BLG cannot be increased to satisfy pharmaceutical requirements then other expression systems may have to be considered. Bovine BLG has already been synthesised in *E.coli* where it accounts for approximately 15% of the total cell protein [180]. Most of the protein that is synthesised is found in an insoluble form which is then solubilised in GuHCl. BLG isolated in this manner appears to be acid stable suggesting that it renatures into its native conformation. It may also be possible in the future to generate transgenic cattle that can synthesise the engineered form of the protein. Transgenic mice have already been created that successfully express ovine BLG in their milk [181]. The use of such an expression system would supply almost limitless amounts of protein that would be easy and cheap to purify.

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