Analysis of Proteolytic Enzyme-Derived Fragments of Murine Alpha-Fetoprotein

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Abstract

The purpose of the present study was (i) to generate proteolytic enzyme-derived fragments of murine alpha-fetoprotein (AFP); (ii) to characterize some of their physicochemical and immunological properties; and (iii) to isolate the fragments from proteolytic digests of mouse AFP. Initial experiments involved the development of a protocol utilizing microgram quantities of mouse AFP to determine the optimum conditions for the limited proteolytic digestions of AFP with various proteases. Detailed SDS-PAGE analysis of the pattern of peptide bands generated by the proteolytic digestions of AFP were found to be characteristic of the protease and were highly reproducible. Significant differences in digestion patterns produced by the limited proteolysis of AFP performed in non-denaturing versus denaturing (containing 0.1% [w/v] SDS) buffers were apparent. Proteolysis under denaturing conditions was most efficient and yielded smaller peptide fragments of narrower molecular weight range. Similar findings were observed for larger scale digests using milligram amounts of AFP. Some of the cleavage products were found to possess antigenic properties of the parent AFP molecule as demonstrated by immunoblotting experiments. Attempts were made to isolate individual AFP peptides from digests of 20-40 mg of the glycoprotein. Various protein separation techniques were employed. The experiments involved gel filtration chromatography, reverse-phase high performance liquid chromatography and successive zone elution by preparative SDS-polyacrylamide gel disc electrophoresis. Only partial separation of the proteolytic fragments was accomplished with these methods. In addition, preliminary experiments were performed which involved testing the immunoregulatory activity of synthetic AFP peptides on an <u>in vitro</u>

lymphocyte reaction known to be sensitive to AFP-mediated suppression, namely the Con-A induced proliferation of Lyt 1^+ 23^- thymocytes. Anti-proliferative activity was observed with one of the peptides, AFP-34/48, which represents an amino acid sequence near the N-terminus of the parent molecule. The information obtained from the above studies represents the groundwork for future investigations of the structure-function relationship of mouse AFP.

Sommaire

Le but de cette étude visait à (i) générer par digestion enzymatique des fragments protéolytiques de l'alpha-fétoprotéine murine (AFP), (ii) d'en caractériser leurs propriétés physicochimiques et immunologiques, et (iii) d'isoler ces fragments. Les expériences ont d'abord impliqué la mise au point des conditions optimales pour produire des digestions protéolytiques partielles de l'AFP par diverses protéases à partir de quantité minimale d'AFP. L'analyse des fragments obtenus par cette digestion par électrophorèse (SDS-PAGE) a révélé des bandes différentes et a permis de démontrer que chaque protéase créait une empreinte unique et constante des fragments. Lorsque l'AFP fut soumis à des digestions partielles en conditions dénaturantes (0.1% [w/v] SDS) ou non-dénaturantes, des différences marquées des empreintes furent trouvées. Les digestions en conditions dénaturantes furent les plus productrices: de plus petits fragments à l'intérieur d'un écart de poids moléculaires plus petit furent générés. Des résultats semblables furent répétés lorsque l'on utilisa une quantité plus élévée d'AFP (milligrammes plutôt que des microgrammes). Quelques uns des fragments possèdent des propriétes antigéniques de la molécule mère d'AFP lorsqu'étudiés par technique de buvardage (immunoblotting). Des essais afin d'isoler les peptides individuels d'AFP furent entrepris à partir de 20-40 mg de la glycoprotéine. Des techniques variées de séparation furent employées à cet escient. Ces expériences ont impliqué la filtration sur gel, elutions de zones successives par électrophorèse préparative dans un gel cylindrique de polyacrylamide avec SDS, et la chromatographie liquide de haute performance en phase renversée. Ces techniques différentes n'ont mené qu'à une séparation partielle des fragments protéolytiques. Par ailleurs, des expériences préliminaires

furent entreprises afin de tester l'activité immunorégulatrice de peptides synthétiques d'AFP. La prolifération des thymocytes Lyt $1^{+}23^{-}$ induits par Con-A est connue comme étant supprimée par l'AFP. Les peptides différents d'AFP ont donc été étudiés pour cette fonction. Un des peptides, AFP-34/48, possédait une activité anti-proliférative. Ce fragment constitue une suite d'acides aminés de la séquence N-terminale de la molécule mère d'AFP. Les informations obtenues à partir de ces études auront servi d'amorce dans l'évaluation du rapport structure-fonction de l'AFP murine.

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List of Abbreviations

Å	Angstrom
AFP	alpha-fetoprotein
anti-BSA	rabbit antiserum against bovine serum albumin
anti-IgG	rabbit antiserum against mouse gammaglobulin
anti-MoAFP	rabbit antisera against mouse alpha-fetoprotein
anti-MoALB	rabbit antiserum against mouse albumin
anti-NMS	rabbit antisera against normal mouse serum
anti-Tf	rabbit antiserum against mouse transferrin
APAGE	alkaline polyacrylamide gel electrophoresis
Bis	N,N'-methylene bisacrylamide
BSA	bovine serum albumin
^{o}C	degree celsius
Ci	curie
CNBr	cyanogen bromide
Con-A	Concanavalin-A
cpm	counts per minute
D	denaturing
ddH_2O	double distilled water
dH_2O	distilled water
DTT	dithiothreitol
endo	endoproteinase
E:S	enzyme:substrate
g	gram
[^{3}H]-TdR	[^{3}H]thymidine
HEPES	N-2-hydroxyethylpeperazine-N'-2-ethane sulfonic acid

HPLC	high performance liquid chromatography
hrs.	hours
HS-PBS	high salt-phosphate buffered saline
Ig	immunoglobulin
IgG	gammaglobulin
INC	inconclusive
Kd	kilodalton
M	molar
mA	milliampere
MAF	mouse amniotic fluid
mg	milligram
mins.	minutes
ml	milliliter
mM	millimolar
MoAFP	mouse alpha-fetoprotein
MoALB	mouse albumin
Mr	relative molecular weight
MW	molecular weight
ND	non-denaturing
NMS	normal mouse serum
PAGE	polyacrylamide gel electrophoresis
PBS	Dulbecco's modified phosphate buffered saline
S4B	sepharose 4B
SDS	sodium dodecyl sulphate
S.E.M.	standard error of the mean
TBS	tris buffered saline
T cell	thymus derived lymphocyte

TEMED	N,N,N',N'-tetramethylenediamine
TFA	trifluoroacetic acid
Tris	tris (hydroxymethyl)-amino-methane
TTBS	tween-20 tris buffered saline
U	unit
µg	microgram
µl	microliter
µm	micrometer
V	volts
vs	versus
v/v	volume/volume
w/v	weight/volume

Introduction

Alpha-fetoprotein (AFP) is a major embryonic serum glycoprotein (1). It belongs to a family of cross-reacting substances present in both fetal and tumor tissues collectively known as "onco-fetal" molecules (2-3). AFP has been identified in all mammals so far studied and homologues have been found in sharks and birds (4). Mammalian AFP is a single polypeptide chain consisting of 4% carbohydrate and an approximate molecular weight of 70 kilodaltons (5). In mammals the primary site of synthesis is the fetal liver (4). AFP synthesized by the fetus during gestation is distributed in fetal serum, amniotic fluid, maternal serum, and cerebrospinal fluid (6). The levels of AFP present in the amniotic fluid and blood reach milligram amounts during perinatal life (4). However, at birth serum AFP levels begin a gradual decline to the low nanogram amounts normally found in the adult (7). Elevated AFP levels, apart from pregnancy (8), are associated with hepatocyte regeneration (9), and a variety of non-malignant (10-13) and malignant conditions (14). AFP is a useful clinical diagnostic marker in hepatocellular carcinoma (14,15) and congenital neural tube defect (13).

AFP possesses similar structural and functional similarities with albumin. These similarities, in addition to the serum concentrations of AFP and albumin being inversely related, have led to the suggestions that AFP may be evolutionally related to albumin (16-19) and that AFP is the fetal counterpart of albumin (20). AFP and albumin are considered to be members of the same small multi-gene family in man and rodents, on the basis of nucleotide and amino acid sequence data (21-23). Their genes are thought to have arisen between 300 and 500 million years ago as the consequence of a gene duplication (19,21,23-26) and are expressed in the yolk sac, liver and fetal gut at different levels (27-30).

Biochemical analysis has demonstrated that AFP and albumin proteins each consist of a single polypeptide chain with apparent molecular weights of 68 to 70 Kd. The slight differences in mobility between these two proteins exhibited on denaturing gels has been accounted for by the fact that only AFP is glycosylated. The potential glycosylation sites found in AFP, but absent in nearly all albumins, are: Asn-X-Ser and Asn-X-Thr. These sites vary not only in number but also in position within the different AFPs which contribute to the vast microheterogeneity, dependent on the tissue type (6).

Examination of the AFP structure by a dark-field electron microscopy with image enhancement was performed on both human and bovine AFPs which were found to be "U" shaped monomers with outside dimensions of 80 Å (31). Each of these molecules seems to be composed of three globular regions called domains. These globular regions appear to be at the vortex as well as the two extremities of the "U" shaped monomer. In addition, both classical optical rotatory dispersions and circular dichroism measurements have suggested that both AFP and albumin possess considerable amount of alpha-helilcal formation (31). Structural information obtained by preliminary x-ray analysis of albumin suggests it is a structure of approximately 140 Å long by 40 Å wide (32).

Further investigation of the structure of these two proteins and their possible correlation to function was made feasible when sequence data became available in the 1970s. The first complete protein sequences to be determined were that of human and bovine albumin (19,33). Cloning and nucleotide sequence determination of mouse (21,25), rat (23) and human (22) AFP have permitted the deduction of the primary amino acid structure of these AFPs. A considerable degree of amino acid sequence homolo

between the AFPs of various mammalian species was noted (65% for mouse/human and 85% for mouse/rat) (22-25). Furthermore, immunological cross-reactivity among AFPs of various mammalian species was also observed (34-36). Immunological cross-reactivity between AFP and albumin has been demonstrated, but only in their unfolded state (37-38), indicating their structural relatedness. There is approximately 35% homology between the primary structure of AFP and albumin. The albumins contain the classical leader sequence required for secretion which consists of 18 amino acid residues, as well as 6 amino acid propeptide which is removed in the Golgi complex (39-40). The AFPs do not possess a propeptide sequence and retain the extra amino terminal residues upon secretion (21).

There are a few differences observed when comparing the amino acid compositions of all the completely sequenced AFPs (mouse, rat and human) and albumins (human, bovine and partially sequenced [85%] rat). The AFPs have more glutamine and the polar but uncharged serine, whereas the albumins instead contain more of the charged amino acid residues aspartate and lysine. The AFPs contain more of the uncharged amino acids isoleucine and methionine and slightly more glycine, whereas albumins contain more valine and slightly more proline. It is unknown at this time how these differences relate to function. Greater conservation of the cysteine residues exists among AFP and albumin in comparison to the percent frequency of any of the other amino acids.

A model depicting the secondary structure of albumin was proposed by Brown (33,41), who recognized that the cysteine residues in human and bovine albumin appeared in a thrice repeated pattern. This gave rise to the concept of a three domain structure having evolved from an original single domain by amplification and divergence (33,42). The sequence of

all of the albumins can be organized to generate three very similar repeated globular units called domains. Each of the three domains consist of two large double loops separated by a small double loop tied together at their base by a pair of adjacent cysteine crosslinks. The loops may correspond to alpha-helical regions based on the known high alpha-helical content of the protein (33). The sequences of all the AFPs can also be formed into the same secondary structure (25), proposed for the albumins. Brown further extended the model by dividing each domain into two "subdomains" since they correspond to predominately alpha-helical regions. Each subdomain consists of three antiparallel alpha-helical rods arranged forming a trough, each about 20 amino acid residues long. The troughs are held together by the cysteine disulfide bonds. The interior region of the trough is predominately non polar, in contrast to the exterior which is mostly polar. The domain is thought to contain a hydrophobic face-to-face arrangement of two subdomains. The hole or slot between the paired domains is therefore proposed to be the hydrophobic ligand-binding site. Law et al. have translated the entire AFP mRNA coding sequence into a corresponding amino acid sequence (21). In addition, they aligned the disulfide bridges of cysteine residues in mouse AFP in the same manner as has been described by Brown for bovine serum albumin (BSA) and human serum albumin (33,41). The resulting structure revealed that the amino acid sequence of AFP was divided into three domains of sequence homology, analogous to the domains of albumin. It was found that 15 disulfide bridges can be formed throughout the AFP molecule as compared to the 13 noted in BSA. In addition, the cysteine residues within the polypeptide chain of AFP occurred at almost identical sequence positions to those observed in albumin. This permitted formation of the same disulfide

bridges in the secondary structure of AFP. The active binding sites in mammalian AFPs have not yet been well characterized. Results of experiments on the intact protein bovine serum albumin and isolated fragments corresponding to domains have allowed the localization of binding sites to specific domains (43) of the parent molecule. These investigations have demonstrated that the protein domains are functionally distinct and that the generation of functional diversity within the protein is responsible for the selective pressure to maintain amplified domains.

Cloning and sequencing of the single copy genes of mouse AFP (19) and rat albumin (23) revealed the genetic basis for the thrice repeated structure of both AFP and albumin. Both genes consist of 15 exons separated by 14 intervening sequences (18). In mouse, the AFP gene (on chromosome 5) is 22 kilobases (kb) of DNA in length, of which only 2.2 kb are required to encode the mature mRNA (18). The albumin gene is situated 14 kb upstream of the AFP gene in the same transcriptional orientation (18), but is only 18 kb long. In addition, a small repeated sequence consisting of 27 bp has been identified in 59 positions distributed evenly throughout mouse AFP (44). The 27 bp consensus sequence possesses a preponderance (69%) of A and G nucleotides, which upon translation corresponds to a large number of glutamic acid and lysine residues. This is consistent with the high levels of these two residues observed in the amino acid composition of AFP. Similar analysis performed on rat albumin messenger RNA sequence was also observed to contain a consensus sequence resembling that for mouse AFP.

AFP and albumin not only share considerable structural features, but also have some functional properties in common. It has been suggested that the high concentrations of AFP and albumin in plasma help control the

osmotic pressure of the intravascular fluid (6,45). Both AFP and albumin serve transport and absorption roles for many endogenous and exogenous molecules (6,46-50). Examples include fatty acids, bilirubin, steroids, indole and some divalent cations. Unlike albumin, AFP is a glycoprotein and, in addition, binds lectins (6). Strong and specific binding of estrogens has been observed only by rodent AFP (51-53) and is believed to be a mechanism to protect rodent brain from effect of excessive amounts of circulating estrogens (53). In addition, AFP, contrary to albumin, has been demonstrated to possess the capacity to exert strong and highly selective immunoregulatory activity in vitro (30,54-58) and in vivo (59-60).

Thus, AFP and albumin have several structural and functional similarities. However, a major functional difference existing between these two proteins is the fact that only AFP possesses immunoregulatory properties. A considerable amount of microheterogeneity has also been observed with AFP. Molecular variants of AFP have been described on the basis of size, charge, and lectin-binding (61). The structure (62-63) and carbohydrate moiety (64) of AFP contribute to its microheterogeneity. Such microheterogeneity may be of potential relevance to the immunoregulatory properties associated with AFP.

The purpose of the present investigation was (i) to generate proteolytic-enzyme derived fragments from murine AFP, (ii) to characterize some of their physicochemical and immunological properties, and (iii) to isolate fragments from proteolytic digestions of mouse AFP.

Literature Review

I Immunoregulatory Properties of AFP

The possible biological functions of AFP that have been suggested include (i) serving as a plasma carrier molecule for certain endogenous and exogenous substances (for example, fatty acids, hormones, bilirubin and some divalent cations) (6,46-49); (ii) maintaining of fetal osmotic pressure (6,65); (iii) regulating of the protein synthesis in the fetal stage (66); and (iv) exerting certain selective immunoregulatory activities (30,67-68). The implication that AFP may have an immunoregulatory function is of great potential importance in the maternal-fetal relationship, the immune capabilities of the fetus and newborn, and in certain non-malignant and malignant diseases associated with elevated AFP levels (67).

Investigations in both murine and human systems have demonstrated that AFP is capable of exerting selective (30,56,58,69) suppressive activity on: T cell-dependent antibody synthesis (54,67,70-71), T cell mitogen reactivity (54-55,62,68,71-81), T cell allogeneic and autologous reactivity (55,57,68-69,71,75-76,78,80,82-84), generation of allogeneic cytotoxic T cells (69), and lymphokine stimulated natural killer activity (85). Studies involving the measurement of immune parameters in normal mice injected with purified murine AFP (59-60) demonstrated that it can exert regulatory influences on the immune system in vivo. It was shown that AFP-treated mice had suppressed antibody responses (59) and a significantly altered natural history of Moloney sarcoma virus-induced tumors (60). In contrast to AFP's immunosuppressive activity, AFP has also been observed to exert an immuno-enhancing activity, particularly when AFP is tested on non-Ia associated immune reactions (56) and on bone marrow cells (86).

Evidence based on several findings has strongly suggested (58) that a primary target for AFP mediated suppression is the function of certain Lyt $1^{+}23^{-}$ T cells. It has also been suggested that AFP may be able to mediate suppression by one or more of the following mechanisms: (i) through a direct anti-proliferative effect (54,56-57,87); (ii) via the induction of suppressor cells (88-90); and (iii) by modifying the expression of Class II antigens on accessory cells (91-92).

The effect of AFP on experimental autoimmune disease has been studied in rats (93) and mice (94). For example, a recent study investigating the effect of AFP on experimental autoimmune myasthenia gravis induced in mice by passive transfer of human myasthenic immunoglobulin was performed (95). This study demonstrated that mouse AFP, when used to appropriately pre- and post-treat the recipient animals, provided significant protection against the disease (95).

II Microheterogeneity of AFP

Molecular variants of AFP have been identified in both rodent- and human-derived AFP preparations, based on differences of size, charge, or lectin-binding (61). Molecular weight heterogeneity of human AFP has been demonstrated by electrophoresis under non-denaturing conditions and is caused by the formation of AFP oligomers (dimers [5,96] and trimers [96]). Their formation appears to be dependent upon intermolecular disulfide bonds since this phenomenon is abrogated upon treatment of AFP with disulfide-reducing reagents (2-mercaptoethanol or dithiothreitol) (5,96). The degree of oligomer formation appears to be dependent upon the degree of manipulation of the AFP preparation, for example lyophilization or freezing and thawing (5). Some size heterogeneity in AFP of several species has also been demonstrated by SDS-PAGE or electrophoresis in gradient pore-size

polyacrylamide gels (61). Differences of 1 to 2 Kd in apparent molecular weights have been observed and have been suggested to be a consequence of either additional peptide on the amino-terminal end of a subspecies of AFP molecules or variations in carbohydrate composition of AFP molecules (61). For example, it has been reported that mouse AFP consists of one molecular size population of 72 Kd or two of 72 and 73 Kd (97) by SDS-PAGE. In contrast, only one molecular size population of human AFP with a molecular weight of 70 Kd was observed (5).

Lectin-binding heterogeneity of AFP has been demonstrated to various extents in rat, mouse and human AFP preparations (61). Differences in lectin-binding of AFP derived from sera of hepatoma patients to the plant lectin concanavalin-A (Con-A) have permitted the identification of two variants of AFP, namely Con-A reactive and Con-A non-reactive AFP (98-99). These variants have also been observed in mouse AFP derived from amniotic fluid and from newborn serum (61). Recent studies involving lectin binding have also demonstrated that molecular variants of AFP might be associated with different pathological processes (100) and may be of potential diagnostic importance (100).

Analysis of charge variants of AFP has been accomplished by extended electrophoresis in agarose gels (71), polyacrylamide gel electrophoresis (97,101-102), immunoaffinoelectrophoresis (97,103), isoelectric focusing (63-65,104), and ion exchange chromatography (65,103-107). Charge heterogeneity of AFP may result from differences in the polypeptide composition (62-63), ligand-binding (61) and/or differences in the carbohydrate moiety (64). Charge molecular heterogeneity has been identified in rodent- and human-derived AFP. The sialic acid content has been found to be only partially responsible for the charge heterogeneity of

AFP, since desialyation of the AFP did not result in its complete loss of heterogeneity (63-64,71). Some investigators have suggested that such residual charge heterogeneity depends upon variation in primary structure of the molecule (62-63) rather than its conformation.

Correlations have been made with the quantity of individual variants of human AFP to various physical and pathological conditions, and differences in biological activity (61-62,105,108). Some investigators have examined the role of the sialic acid residues of human and murine AFP with respect to the immunosuppresive activity exerted by AFP on certain immune reactions in vitro. The role of these residues is a contentious issue. Zimmerman et al. (70) have reported that complete desialyation of murine AFP resulted in the loss of its immunosuppressive activity. In contrast, Lester et al. (62) have reported that asialyated human AFP retained its immunosuppressive activity. They suggest another post-synthetic modification than that of the sialic acid residues present in human AFP regulates its immunosuppressive activity (109). Recent investigations with mouse AFP in the murine system have demonstrated that its immunosuppressive activity is localized in one particular molecular variant. This activity cannot be accounted for by sialic acid residues (110).

III Biologically Active Peptides

Evidence has accumulated in the past few decades that small peptides (about 2-40 amino acid residues), often the products of processing of larger precursor proteins, constitute an important class of modulators of biological function (111). For example, many small peptides serve as hormones in the endocrine system, and as modulators or neurotransmitters in the central nervous system. In addition, some of the diverse biological

functions that are thought to be regulated or modulated by such small peptides include enzyme inhibition, sexual maturation and reproduction, blood pressure, gastrointestinal digestion, glucose metabolism and storage, thermal control, satiety, analgesia, and learning and memory (111).

The majority of small peptides involved in such systems generally interact with specific membrane components (e.g. receptors) which in turn initiate a biological response in the target cell (111). As yet, the details of the mechanism(s) of action of most peptides are still unknown.

Important advances in the synthesis of biologically active peptides have occurred since the first synthesis of the hormone oxytocin in 1953 (112). The total synthesis of some complex peptides has been rendered possible with the introduction of the solid-phase method by Merrifield in 1963 (113) and repetitive methods in solution. Only a few of the synthetic complex peptides such as human insulin and human gastrin have full biological activity (114). However, it has been demonstrated that some synthetic analogs possess higher biological activity when compared to the parent molecule (115-116).

The biological activity of some proteins can be localized to a particular peptide sequence or region of the parent molecule. The sequences of certain peptide hormones have provided the basis for their synthetic production and, in some cases, for the synthesis of peptide analogs. This has also led to a knowledge of the functions of particular regions of the molecule. For example, the synthetic pentapeptide, Thymopentin is homologous in composition to that of thymopoietin (thymus hormone) represented by residues 32-36. Similarly, the synthetic pentapeptide Splenopentin is homologous in composition to that region of splenin (product found in spleen and lymph node), represented by residues

32-36. These pentapeptides have been shown to reproduce the biological activities of thymopoietin and splenin, respectively (117-118). In addition, Cianciolo et al. (119) have recently synthesized a heptadecapeptide (CKS-17). This peptide is homologous to a highly conserved region of the immunosuppressive retroviral protein p15E (120-124), envelope proteins of human T cell lymphotropic viruses I, II and III, and to endogenous C-type human retroviral DNA (125-128). CKS-17 was found to possess immunosuppressive activity in vitro which was evident only when CKS-17 was covalently coupled to the carrier protein bovine serum albumin (BSA) (119,129-132). Cianciolo et al. suggested that the biological activity of the synthetic peptide might be dependent on a particular conformation that is conferred on the peptide molecule by coupling it to a carrier protein (119). Furthermore, certain fragments of BSA obtained by proteolysis (131,133) and limited proteolysis (43,134) were demonstrated to have retained some biological features of the parent molecule such as ligand-binding. Amino acid analysis, molecular weight estimation and terminal sequences of proteolytic fragments of BSA helped in determining their location within the BSA molecule according to the sequence proposed by Brown (41). This permitted the correlation of the properties demonstrated by the fragment with a particular region or site of BSA. This allowed the identification of active-sites of BSA and their locations within the molecule relative to one another.

IV Analysis of Structure-Function Relationship of a Protein

Many biological investigations involving analysis of the structure-function relationship of a protein have focused on the production of peptides derived from the protein under study (135). Biophysical methods have been used to study the conformation of the produced peptides (111) and

the possible relation it may have to the peptide fragment and its parent molecule. In addition to the biophysical methods such as x-ray crystallography and spectroscopic methods (infrared, optical rotatory dispersions, circular dichroism and nuclear magnetic resonance), theoretical calculations and modern computers have also been used to examine peptide conformations (136-137).

Synthetic approaches to peptide production include solution and solid phase techniques, and the use of recombinant DNA technology. There are limitations in the production of peptide fragments by synthesis and recombinant DNA technology. Synthetic peptides composed of only up to 30-40 amino acid residues can be synthesized while still maintaining a high degree of purity (114). Recombinant technology is limited in that this technique is not of use in synthesizing peptides containing uncommon amino acids such as D-amino acids andpeptides with blocked N^{α}-NH_2 groups (114). The degradative approaches to peptide production involve the controlled digestion of large amounts of purified protein, producing a pool of peptide fragments (135). The actual digestion can be accomplished using either proteolytic or chemical techniques (135). Many of the chemical cleavage procedures devised suffer from the disadvantage of poor yield of peptide bond fission as well as the occurrence of undesired reactions in side chain groups (138). In contrast, fragments obtained by proteolysis or limited proteolysis undergo minimal chemical damage in that they have intact disulfide bonds and unaltered amino acid side chain groups (43,139). They resemble more closely the native molecule than fragments prepared by harsher chemical cleavage techniques (43,139) and would be expected to retain many of its structural features.

A. Proteases and Limited Proteolytic Digestion of a Protein

Proteolytic enzymes called proteases are enzymes that catalyze the hydrolysis of peptide bonds. Most proteases also catalyze the hydrolysis of ester bonds. In the case of a peptide bond the reaction products are a carboxyl component and an amino component of the substrate, while the hydrolysis of an ester bond results in the formation on an acid and alcohol. Proteases are involved in many biological control mechanisms. These include the complete breakdown of a protein in digestion and limited proteolysis (selective proteolytic cleavage of a peptide bond) of a zymogen (a protein inactive precursor) (140). In addition, proteases have been utilized in attempts to delineate certain aspects of three-dimensional conformation and structure-function relationships in proteins (141). Proteases can be subdivided relative to their mechanisms of action or their function. Five major classes of proteases yielded when thay are classified according to their mechanism of action (142). These are: (i) **serine proteases** which contain an active-site serine residue (143), (ii) **sulfhydryl or thiol proteases** which contain an active-site cysteine residue, (iii) **metalloexopeptidases** which contain active-site metal ions and hydrolyze peptide bonds near the ends of polypeptides or peptides, (iv) **metalloendopeptidases** which also contain active-site metal ions but hydrolyze peptide bonds in the interior of polypeptide chains, and (v) **carboxyl (acid) proteases** which function only in acid environment (pH optimum 2-3). Proteases vary greatly in their degree of substrate specificities. Some proteases are of high specificity. Their specificity is restricted to the cleavage of peptide bond(s) at only certain amino acids. This is an advantage when limited proteolysis of a given protein is desired. Proteases of high specificity include endoproteinase Glu-C (144),

trypsin (139), endoproteinase Arg-C (145) and endoproteinase Lys-C (146). Other proteases are of lower or more general specificity, that is they cleave peptide bonds at a wide variety of amino acids. Bromelain, pronase, chymotrypsin A_4, elastase, papain and thermolysin (139) are included amongst these.

Limited proteolysis of a protein involves restricting its proteolytic cleavage to a few of the many potential cleavage sites. Consequently, large peptide fragments may be produced. The optimal procedure and conditions for a particular proteolytic digestion clearly depends on the protease and the substrate concerned, and on the degree of cleavage required. In addition, for any particular proteolytic digestion the following four variables must be considered. These are: (i) buffer, (ii) enzyme:substrate ratio, (iii) temperature, and (iv) period of incubation. Variations of these four parameters will aid in obtaining the required cleavage and the correct combination to permit the desired limited proteolysis of the protein. However, one must also consider the protease to be used, its specificity, and where known, the chemical composition and physical condition of the protein. These are important in the extent of digestion. For example, if a native protein structure is digested it is probable that cleavage will be restricted because of the manner in which the molecule may be folded. Consequently, this may cause some of the proteolytic cleavage sites not to be as accessible as others for recognition by the protease. This notion is useful in limiting the proteolysis of a protein and may also be of advantage in isolating fragments with a particular biological activity since disulfide bonds may be retained. Some proteases retain some or all of their activity under certain denaturing conditions such as in low concentrations of urea,

guanidine hydrochloride, or sodium dodecyl sulfate. These are reversible by simple dialysis. Such conditions are of importance in proteolysis for they will permit unfolding of the protein and allow exposure of some cleavage sites which would otherwise not be accessible to the protease under non-denaturing conditions. Proteolysis of a protein in non-denaturing versus denaturing conditions may therefore result in different digestion patterns.

Since proteases function over a narrow range of pH, one method of terminating the reaction is to alter the pH to far outside the protease's functional pH range (139). If, however, the pH of the solution is subsequently restored to the initial value (that is, within the protease's functional pH range), the protease may recover its activity and an alternative method may be required to terminate the reaction. Depending on the protease being employed in the digest, proteolysis can also be terminated by addition of the appropriate enzyme inhibitor (139). Another method of terminating proteolysis is by boiling the reaction mixture. This will destroy the protease (139). However, vigorous treatments of this type are permissible only if they leave the substrate unaffected.

B. Analysis of Albumin's Structure-Function Relationship

Albumin is known to bind a wide variety of organic and inorganic ligands, and functions as a plasma carrier molecule for certain endogenous and exogenous substances (50). It probably also serves as a mechanism in regulating the blood level of certain drugs and metabolites (50). Studies were carried out to determine what molecular parameters of albumin were required for its binding to various ligands. Bovine serum albumin (BSA) was used in these studies and the approach taken to analyze albumin's structure-function relationship involved the controlled degradation of

native BSA. This was accomplished by limited proteolysis of the native BSA molecule. Fragments of BSA isolated from limited tryptic digests (134) and limited peptic digests (147) were obtained and used in the study of the location (134,147) and properties of binding sites (43,131-133) and for the determination of other structural, functional and immunological features of the parent molecule.

A fragment of BSA prepared by tryptic hydrolysis (132) or peptic hydrolysis (133) and representing the carboxyl-terminal portion of the molecule was shown to retain strong binding sites for both octanoate and L-tryptophan, unlike a similar fragment prepared by cyanogen bromide cleavage (132). Several fragments of BSA have been isolated following limited tryptic digestion (134) and limited peptic digestion (147). Amino acid composition, molecular weights and terminal sequences of such proteolytic fragments served to determine their location in the amino acid sequence of BSA derived by Brown (41). Localization of the fragments permitted the correlation of the properties demonstrated by the fragment with a particular region in BSA. This permitted identifying active areas of BSA and their locations within the molecule relative to one another. For example, the binding site for indole was deduced to be located in the amino-proximal (first) domain, the binding site for bilirubin in the middle (second) domain and the high affinity binding site for long chain fatty acids in the carboxyl-terminal (third) domain (43).

Circular dichroism studies were performed with albumin and the proteolytic fragments of BSA (43). These studies demonstrated that the conformational pattern of albumin (68% α helix and 18% β structure) is substantially retained by individual fragments indicating that secondary configuration is locally determined and is not destroyed during the

cleavage process nor during fragment purification (43). This supports the premise that proteolytic fragments may be expected to demonstrate properties similar to those of the parent molecule. These proteolytic fragments were also used in investigating the mechanism of protein folding in BSA (148). These investigations demonstrated independent folding of structural regions and support the concept of multiple sites for nucleation and growth of native structure as proposed by Wetlaufer (108). Analysis of the antigenic structure of BSA has been accomplished by studying the antigenicity of proteolytic fragments of BSA (149-151). It was found that fragments from both the amino-proximal terminal and the carboxyl-terminal of the molecule account for nearly all of the antigenic reactivity of the native protein (149-151). This led to the conclusion that native albumin possesses identical repeating antigenic sequences. In addition, immunogenic properties of a peptic fragment of BSA, also produced by limited proteolysis, has been investigated (152).

V Techniques for the Isolation of Peptides from a Peptide Mixture

A. Conventional Chromatography

Various techniques are used for the separation of peptide mixtures derived from proteolytic or chemical cleavage. Among these are gel filtration and ion exchange chromatography, or a series or combinaton of these techniques. To date, the only method that remains complementary to HPLC is gel filtration chromatography performed on dextran- or polyacrylamide-based supports (153). In this technique, peptides are chromatographed according to their molecular weights. Gel filtration chromatography has several advantages. For instance, a wide selection of support materials can be used and are commercially available. Also, the chromatography can be performed over a wide range of pH and with eluents

which may contain a large percentage of organic acids or solvents. Well packed columns are sufficiently stable to permit repeated use, and yields are normally respectably high (153). However, gel filtration chromatography is not a rapid process, but was the method most conveniently employed to fractionate large peptides. Some success has been achieved in the fractionation of large denatured peptides of a peptide mixture when employing gel filtration in the presence of high concentrations of organic acids (e.g. 1 to 4 M formic acid) or denaturants (e.g. urea, guanidine-hydrochloride, SDS or chaotropic salts) (154). Unfortunately, most of these solutions are quite harsh and may be harmful to the peptides. In addition, many of these solutions absorb at the wavelengths most appropriate for the detection of peptides (206 to 230 nm) (154). However, a crude chromatographic profile may be obtained by following the 254 nm and 280 nm absorbances, which depend on the presence of aromatic amino acid residues which are not uniformly distributed in the peptides (154). Using a weak phosphoric acid solution as a solvent system, however, will allow the UV detection of protein or peptide at 206 nm and higher. There are other difficulties involved as well, such as the insolubility of some large peptides in phosphoric acid, and the solvent is not completely volatile and requires a cumbersome desalting procedure. Gel filtration at high pH may be carried out with volatile buffers, but this is limited by peptide insolubility and poor chromatographic characteristics.

Separation of a peptide mixture that could not be accomplished on the basis of molecular weight alone required another method of fractionation, often employed was ion exchange chromatography. Such as cation-exchange chromatography on sephadex or cellulose in the presence of 8 M urea was accessible (153). A combination of these techniques has been used for the

successful isolation of BSA fragments from limited tryptic (134) or limited peptic (147) hydrolysis. However, large initial quantities (about 1 gram) of BSA were required in the digestions for successful fractionation of the peptide mixtures in order to recover workable amounts of isolated proteolytic fragments. However, the efficiencies of such systems as measured by the quantity of time and frustration involved in developing a successful chromatogram were low when compared with some of the present day techniques such as reverse-phase HPLC (153).

Some of the different methods available and used for the preparative fractionation of small peptides (<30 amino acid residues) included ion exchange chromatography performed on polystyrene-based supports, paper chromatography, and paper electrophoresis (153). However, the peptide recoveries from any one of these methods are not in any way quantitative and the limited power of resolution of each necessitates the use of more than one technique for the isolation of peptides from complex mixtures. As a consequence, final yields are low.

B. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) has permitted the analytical separation of medium to large peptides. The technique of PAGE include separations based on intrinsic charge to mass ratio of the protein (155), apparent molecular weight in the presence of SDS (156), and isoelectric focusing (157). This technique has a great power of resolution for quantities of peptides ranging from 5 pg to several milligrams. SDS-PAGE has permitted the analysis of peptide fragments produced by either limited proteolytic or chemical cleavage of proteins, and allows peptide mapping which is particularly useful for investigating similarties between samples.

Preparative PAGE for the isolation of proteins or peptides can be performed by (a) excision of protein or peptide zones (bands) and extraction of their content from gel slices (158) or (b) successive zone elution (159). The former has been used for the isolation of several peptides or proteins in only sub-milligram quantities and involves the excision of individual bands from rod gels or slab gels. A sample containing several milligrams of peptides is loaded onto the gel using a discontinuous buffer (the sample capacity of the gel depends on its thickness). It is then electrophoresed and the bands are located and excised. Individual gel slices are then eluted by simple diffusion (160-161) or by electrophoretic elution (158,162-163). Successive zone elution is used for the isolation of a single peptide or protein from a mixture in amounts of a few milligrams or more. Successive zone elution is most successful when using a specialized polyacrylamide gel apparatus which permits the collection of peptide or protein zones as they elute from the base of a large-scale gel column (159,164), therefore the name preparative polyacrylamide gel disc electrophoresis. However, there are some disadvantages to successive zone elution. These include excessive dilution of eluted peptides or proteins, poor resolution of closely migrating peptides or proteins, and extended electrophoresis times for peptides or proteins of low mobility. The technique of successive zone elution can be also performed with a SDS-discontinuous buffer system, which will permit separation on the basis of apparent molecular weight.

C. Reversed-Phase High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) has become a powerful and important tool in protein and peptide chemistry. HPLC supports an entire range of chromatography methods which include: (i) size exclusion

separations based on molecular weight differences, (ii) ion exchange separations based on charge differences, and (iii) reverse-phase separations based on hydrophobicity differences (165). Advances in the instrumentation of HPLC has permitted the introduction of many new separation methods, both at the analytical and preparative scale, resulting in significant improvements in the capability to purify proteins and peptides. For functional and structural analysis HPLC has several attributes and advantages over conventional chromatography of peptides. Examples are rapidity, versatility, sensitivity, high resolving power, higher recoveries and the fact that samples have been demonstrated to retain both immuno- and bioactivity (166-167). This makes it a valuable tool for the purification of biologically active peptides or fragments for funtional studies.

In particular, reverse-phase HPLC has demonstrated good and reproducible separations of various standard peptides and is the most widely used method for the separation of complex peptide mixtures. In addition, the sensitivity and the easy recovery of the peptides for characterization (168), amino acid analysis (169), sequencing (169) and peptide mapping of proteins (170-172) have contributed to its popularity. Reverse-phase, as opposed to normal-phase, chromatography is performed on a non-polar stationary phase and the mobile phase is polar. The solutes bind to the surface of the column packing by non-polar or hydrophobic interactions which determine the migration velocity along the stationary phase. The mobile phase represents a gradient of increasing volume ratio of organic modifying solvent which is then run through the column. The first solutes to emerge from the column are those which are most weakly interacting with the hydrophobic stationary phase. Therefore, selectivity

in these separations is essentially based on differences in relative hydrophobic contact areas established between the solutes and the stationary phase. Generally, stationary phases consisting of column packing coated with C_{18} or C_8 alkane chains are used, although other types of coatings are available (173). Separations can be influenced by the numerous experimental options for controlling selectivity and recovery which can be exploited. These options include the organic phase modifier, water content of the mobile phase, ionic strength, pH, temperature, the capacity for ion-pairing, as well as ion exchange effects. The choice of mobile solvent is critical and many different solvent mixtures and chromatography conditions have been employed for particular separations (173). A certain few solvent systems have been widely used. One of the more popular solvent systems consists of 0.1% (v/v) trifluoroacetic acid (TFA) in water and a linear gradient of acetonitrile containing 0.1% (v/v) TFA (154). The method of reverse-phase HPLC is a very powerful tool for the resolution of peptides of up to about 50 amino acids in length, but is less suitable for large hydrophobic peptides and proteins. However, methods to rectify this are presently being investigated.

Materials and Methods

I Animals

Adult Swiss male and female mice, purchased from Charles River, St. Hyacinth, Canada, were maintained and bred in our own facilities. Pregnant Swiss mice were used as a source of amniotic fluid. Adult male CBA/J mice, purchased from Jackson Laboratories, Bar Harbor, Maine, (8-10 weeks old) were used in cell culture experiments.

II AFP Source Material

Mouse AFP was purified from mouse amniotic fluid (MAF) collected (as previously described [174]) from Swiss mice in the late second and early third trimester of pregnancy. The collected MAF was pooled, centrifuged and stored at -20°C.

III Preparation of Antisera

Antisera against mouse alpha-fetoprotein (anti-AFP), normal mouse serum (anti-NMS) and the mouse serum proteins transferrin (anti-Tf), albumin (anti-MoALB), and gammaglobulin (anti-IgG) were produced in rabbits which were immunized with the relevant antigens according to a modified procedure of Harboe and Ingild (175). Adult male rabbits (New Zealand Albinos) were injected at multiple intra-muscular sites with a total of 250 µg (1.0 ml) of pure protein (AFP, Tf, MoALB, or IgG) emulsified in an equal volume of complete Freund's adjuvant. Antiserum to normal mouse serum was produced by injecting 5 mg (1.0 ml) of NMS emulsified in an equal volume of complete Freund's adjuvant. Booster injections of protein or NMS identical to the initial doses (emulsified in equal volume of incomplete Freund's adjuvant) were administered two weeks post-immunization. Beginning two weeks after the booster injections, the rabbits were bled from an ear vein every 10-14 days for a period of 2-3 months (at which time

another booster injection was administered). The serum was isolated from the blood by decanting off the serum after clotting, and centrifuging it at 2000 rpm for 20 minutes. The IgG fractions of the rabbit antisera were isolated by a double precipitation with 18% sodium sulfate (Na_2SO_4). The antibodies were demonstrated to be monospecific for their relevant antigen(s) by Ouchterlony immunodiffusion and immunoelectrophoresis.

IV Purification of AFP by Antibody-Agarose Affinity Chromatography

A. Preparation of Substituted Affinity Matrices

The purified IgG antibody preparation of rabbit antisera against normal mouse serum (anti-NMS) was conjugated to cyanogen bromide-activated sepharose 4B (CNBr, Sigma Chemical Co., St. Louis, Missouri; S4B, Pharmacia Ltd., Dorval, Quebec) with some modification to the method of Cuatrecasas et al. (176). Sepharose 4B was washed extensively with distilled deionized water (dH_2O) on a Buchner funnel under suction. The dried gel was weighed and mixed with an equal volume of water resulting in a gel slurry. CNBr (15 g CNBr per 30 g of S4B) was then added to the gel slurry and was allowed to dissolve by gentle stirring. The pH was immediately adjusted and maintained at 11 by titration of 50% (w/v) NaOH into the gel slurry. The temperature of the reaction mixture was maintained at 20°C by the addition of crushed ice. As the reaction neared completion, the pH of the mixture stabilized at 11. Once the reaction was completed, the activated S4B was washed with 3 liters of cold dH_2O and 2 liters of 0.1 M $NaHCO_3$.

The IgG preparation of anti-NMS was adjusted to a concentration of 5 mg/ml in 0.1 M $NaHCO_3$ - 0.5 M NaCl. The antisera preparation was added to the activated S4B (1 g protein per 30 g of activated S4B) and the mixture was stirred at 4°C for 24 hrs. The gel was then washed with 2 liters of 0.5 M NaCl in phosphate-buffered saline (high salt PBS =

HS-PBS). The excess active groups were blocked by alternative washings with 0.2 M glycine-HCl pH 2.8, and 1 M ethanolamine. The antibody-agarose gel was then extensively washed with HS-PBS, packed into chromatography columns (40-60 g of gel per column), and stored at 4°C until employed in the purification of AFP.

B. Antibody-Agarose Affinity Chromatography

Mouse AFP was purified from mouse amniotic fluid (MAF) by antibody-agarose affinity chromatography, performed at room temperature by passage of MAF over an anti-NMS column (prepared as described above) that was equilibrated with HS-PBS. The MAF (collected and pooled as previously described [174]) was filtered through an AP 25 prefilter (Millipore Corp., Bedford, MA) followed by filtration through an AP 15 prefilter (Millipore Corp.) and then through a 0.45 μm HA membrane filter (Millipore Corp.). Thirty milliliters of filtered MAF (as described above) was gently layered onto the anti-NMS column (25-30 cm bed height antibody-agarose matrix) and was permitted to flow by gravity through the antibody-agarose matrix at a controlled flow rate. Once all of the MAF had entered the antibody-agarose matrix, HS-PBS was gently added to the column. Mouse AFP not retained by the matrix was eluted with 400-500 ml of HS-PBS and collected in 50 ml fractions. Proteins, such as albumin and transferrin, bound by the gel were eluted with 500 ml of 4.0 M guanidine. The column was then regenerated with 1 liter HS-PBS and stored at 4°C in HS-PBS.

Each of the 50 ml fractions were immediately concentrated down to 20 ml in an Amicon Ultrafiltration Cell using a Diaflo Ultrafilter PM-30 membrane (30 000 MW cut-off; Amicon Canada Ltd., Oakville, Ontario) and the concentrate dialyzed against PBS at 4°C for 48 hrs. Prior to dialysis, aliquots from each of the 20 ml concentrated fractions were tested for

presence of AFP and for contamination by other proteins using Ouchterlony immunodiffusion analysis with anti-MoALB, anti-NMS, anti-Tf, anti-IgG and anti-MoAFP. Following dialysis, fractions that contained only AFP were pooled and concentrated (as before) down to a total volume of 10-15 ml and filtered through a 0.45 μm HA membrane. The protein content of the isolated purified AFP preparation was quantified by absorbance at 280 nm with a calculated extension coefficient determined as previously described by Murgita and Tomasi (174).

V Analytical Techniques

A. Ouchterlony Immunodiffusion

Double immunodiffusion in 1% (w/v in PBS) agarose was performed in 60 x 15 mm plastic petri dishes (Fisher Scientific Co., Montreal, Quebec) according to the method of Ouchterlony (177). Among the antisera used in the analysis: anti-MoAlB, anti-NMS, monospecific anti-MoAFP, anti-Tf, and anti-IgG. This technique was used routinely to test for (177) the presence or absence of relevant antigens in AFP preparations and proteolytic digestions of AFP; and (174) IgG fractions of rabbit antisera for their relevant specificities.

B. Immunoelectrophoresis

Immunoelectrophoresis (178) was performed on a plastic frame (plate) holding six 7 1/2 x 2 1/2 cm microscope slides precoated with 0.5% (w/v in dH_2O) agarose (Type I: Low EEO; Sigma Chemical Co.) covered with 1% (w/v) agarose (Type II: Medium EEO; Sigma Chemical Co.) dissolved in Tris-glycine-barbitone sodium-barbitone pH 8.8, ionic strength 0.08 (90.4 g Tris, 112.4 g glycine, 26 g barbitone-sodium and 4.15 g barbitone in 2 liters of dH_2O). The agarose gel once applied to the slides was allowed to solidify at room temperature. The plate was then stored in a humidified

chamber at 4°C for 18-24 hrs. prior to electrophoresis. A template was used to cut antigen wells and antibody troughs into the gel. The agarose was removed from the wells and paper wicks were used to connect the gel to the buffer compartments of the electrophoresis aparatus. The plate was pre-electrophoresed for 15 mins. at a constant voltage of 250 V in the Tris-glycine-barbitone sodium-barbitone buffer pH 8.8, ionic strength 0.08 (prepared as described above). This was followed by application of antigen to the wells and electrophoresis for 2 hrs. at 250 V (at constant voltage). After the termination of the electrophoresis, the agarose was removed from the cut-out troughs and antibodies were applied. The plate was permitted to develop in a humidified chamber at room temperature for 24 hrs., washed in 1% (w/v) NaCl in water for 24 hrs. and air-dried at room temperature for 24 hrs. The slides were then removed from the plate, stained for 5-10 mins. in a solution containing 1% (w/v) thiazine red dissolved in glacial acetic acid:water (1:99, v/v) and destained in a solution of methanol: glacial acetic acid:water (30:10:60, v/v/v), until the background became colorless.

C. Polyacrylamide Gel Electrophoresis

C.1 Alkaline-PAGE

Alkaline polyacrylamide gel electrophoresis (APAGE) was performed in 1.5 mm thick slab gels in the non-dissociating discontinuous buffer system of Davis (155).

The stock monomer solution for both the resolving and stacking gels was prepared as follows: 30 g of acrylamide (BioRad Canada Ltd., Mississauga, Ontario) and 0.8 g of N,N'-methylene bisacrylamide (bisacrylamide; BioRad Canada Ltd.) dissolved in a total volume of 100 ml of double distilled deionized water (ddH_2O). This acrylamide-bisacrylamide stock solution was stored in the dark at 4°C for up to a period of a month.

The resolving gel was prepared by mixing: 15.0 ml of acrylamide-bisacrylamide stock solution, 3.75 ml of 3.0 M Tris pH 8.8, and 9.75 ml of ddh_2O. The mixture was deaerated and chemically polymerized by addition of N,N,N',N'-tetramethylenediamine (TEMED; BioRad, Canada Ltd.) and ammonium persulfate (BioRad Canada Ltd.) to a final concentration of 0.05% (w/v) and 0.08% (w/v), respectively. This yielded a 15% (w/v) acrylamide resolving gel. The stacking gel was prepared by mixing: 1.5 ml of acrylamide-bisacrylamide stock solution (described above), 2.5 ml of 0.5 M Tris-HCl pH 6.8, and 5.9 ml of ddH_2O. The mixture was deaerated and chemically polymerized by the addition of TEMED and ammonium persulfate each at a final concentration of 0.1% (v/v). This yielded a 4.5% (w/v) acrylamide stacking gel. A slab gel prepared in this manner was electrophoresed in a BioRad PROTEAN Dual Vertical Slab Gel Electrophoresis Cell. Pre-electrophoresis of the slab gel was performed at constant voltage of 200 V for 30-45 mins. Protein samples were mixed 1:1 (v/v) with a solution consisting of 40% (v/v) glycerol and 0.1% (w/v) bromophenol blue, in 20-40 µl aliquots containing 10-20 µg of protein. Electrophoresis of the protein samples was conducted for a total period of 6-7 hrs. The samples were electrophoresed at a constant voltage of 100 V through the stacking gel and at 250 V through the resolving gel. Immediately following electrophoresis, the gel was fixed in a solution consisting of 5% (w/v) trichloracetic acid and 5% (w/v) sulphosalicylic acid for 16 hrs., rinsed in dH_2O and stained for 4 hrs. at room temperature in 0.5% (w/v) Coomassie Brilliant Blue R-250 (BioRad Canada Ltd.) and 0.5% copper sulphate in glacial acetic acid: methanol:dH_2O (10:30:60, v/v/v). The gel was then diffusion-destained at room temperature by repeated washing in glacial acetic acid:methanol:dH_2O (10:30:60, v/v/v) until the background became colorless.

C.2 Sodium Dodecyl Sulfate-PAGE

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed in 1.5 mm thick slab gels in the dissociating SDS-discontinuous buffer system of Laemmli (156).

The resolving gel was prepared by mixing: 20.0 ml of the acrylamide stock solution (as described above), 10.0 ml of 1.5 M Tris-HCl pH 8.8, 0.40 ml of 10% (w/v in ddH_2O) and 9.1 ml of ddH_2O. The mixture was deaerated, chemically polymerized by the addition of TEMED and ammonium persulfate into final concentration of 0.1% (v/v) and 0.07% (v/v), respectively. This yielded a 15% (w/v) acrylamide resolving gel. The stacking gel was prepared by mixing: 1.5 ml of acrylamide-bisacrylamide stock solution (as described above), 2.5 ml of 0.5 M Tris-HCl pH 6.8, 0.10 ml of 10% (w/v in ddH_2O) and 5.8 ml of ddH_2O. The mixture was deaerated and chemically polymerized by the addition of TEMED and ammonium persulfate each at a final concentration of 0.1% (v/v). This yielded a 4.5% (w/v) acrylamide stacking gel. Pre-electrophoresis of the slab gel was performed in the apparatus as described above at a constant voltage of 200 V for 30-45 mins. Protein samples were mixed 1:1 (v/v) with an SDS-sample buffer solution consisting of 0.0625 M Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 0.2 M dithiothreitol (DTT) and 0.1% (w/v) bromophenol blue, in 20-40 µl aliquots containing 10-20 µg of protein. The protein samples were heated in a water bath at 100°C for 5 mins., electrophoresed through the stacking gel at a constant voltage of 100 V and through the resolving gel at a constant voltage of 250 V. Electrophoresis of the protein samples was conducted for a total period of 5 1/2 - 6 1/2 hrs. The gel was then fixed, stained and destained as described in C.1, Alkaline-PAGE.

In some cases, as indicated, SDS-PAGE was carried out on a 0.75 mm thick 15% (w/v) acrylamide slab mini-gel in the BioRad Mini-PROTEAN II Electrophoresis cell according to the dissociating SDS-discontinuous buffer system of Laemmli (156) as described above. Pre-electrophoresis was performed at a constant voltage of 200 V for 15-20 mins. Aliquots consisting of 2.5-10 µl of sample were prepared 1:1 (v/v) with SDS-sample buffer solution (also as described above) and heated at 100°C for 5 mins. Electrophoresis of the prepared samples was conducted at a constant voltage of 100 V through the stacking gel and at a constant voltage of 250 V through the resolving gel, for a total period of 45-60 mins. The gel was fixed for 45-60 mins., stained for 60 mins. and destained as described in C.1, Alkaline-PAGE.

C.3 Isoelectric Focusing-PAGE

Isoelectric focusing (IEF) was performed in a 0.75 mm thick 8.5 (w/v) polyacrylamide slab gel containing 2% ampholytes pH range 3.0 - 10.0 (Pharmacia Ltd.) with the buffers as previously described by Righetti and Drysdale (157). The cathode buffer consisted of 20 mM NaOH, whereas the anode buffer consisted of 10 mM H_3PO_4. Pre-electrophoresis of the slab gel was carried out at a constant voltage of 1000 V for a period of 30 mins. Five to ten micrograms of the sample in 40% (w/v) glycerol and 0.1% (w/v) bromophenol blue, in a total volume of 10 - 15 µl, was electrophoresed at a constant voltage of 1000 V for 4 hrs. Immediately following electrophoresis, the gel was fixed, stained and destained as described in C.1, Alkaline-PAGE.

D. Immunoblotting

Antigens were separated by vertical SDS-PAGE on a 0.75 mm thick 15% (w/v) acrylamide slab mini-gel in the BioRad Mini-PROTEAN II

Electrophoresis Cell according to the dissociating SDS-discontinuous buffer system of Laemmli (156) as described in section III A.2. Immediately after SDS-PAGE of the antigen, the gel was equilibrated for 15-30 mins. in transfer buffer pH 8.2 (12.5 mM Trizma base, 96.0 mM glycine and 20% [v/v] methanol). The immobilized matrix employed was Immobilon PVDF Transfer Membrane (Millipore Corp.) which, prior to electroblotting, was wetted in a small volume of 100% methanol for 1-2 seconds. The transfer membrane was then soaked in dH_2O for 5 mins. and was then equilibrated in transfer buffer pH 8.2 as described above. Electroblotting (179) of antigen from the electrophoresed SDS-gel slab onto Immobilon PVDF Transfer Membrane was performed in a Hoefer TE-52 transfer apparatus, with a cooling system, at a constant current of 200 mA in transfer buffer pH 8.2. After electroblotting, the transfer membrane was gently agitated for 1 hr. at room temperature in blocking solution (3% [w/v] gelatin in 20 mM Tris, 500 mM NaCl [Tris-buffered saline, TBS] pH 7.5) to block free binding sites. The transfer membrane was rinsed twice over two successive 10 min. periods with TBS containing 0.05% (w/v) Tween-20 (TTBS) with gentle agitation. The transfer membrane was then gently agitated at room temperature for 5 1/2 hrs. in a 1:500 (v/v) dilution of primary antibody, monospecific anti-MoAFP that had been rendered specific by absorption with equal volume of NMS, in antibody buffer (1% [w/v] gelatin in TTBS). After the transfer membrane was rinsed with TTBS (as described above), it was then incubated at room temperature for 1 hr. with a 1:3000 (v/v) dilution of secondary antibody, BioRad's goat anti-rabbit gammaglobulin alkaline phosphatase conjugate, in antibody buffer. The transfer membrane was rinsed with TTBS (as described above) and was then rinsed in TBS for 5 mins. A modified method of Blake et al. (180) was used for visualization

of the enzyme conjugates. The immunoreactive bands are detected by treating the transfer membrane with the following mixture: 1.0 ml of 15 mg/ml of 5-bromo-4-chloro-3-insolyl phosphate (BCIP) (prepared in 100% N,N-dimethylformamide [DMF]) and 1.0 ml of 30 mg/ml of nitroblue tetrazolium (NBT) (prepared in 70% DMF [v/v] in dH_2O) into 10.0 ml of carbonate buffer pH 9.8 consisting of 0.1 M $NaHCO_3$, 1.0 mM Mg Cl_2. Development of the immunoreactive bands was performed at room temperature for 40 mins. with gentle agitation of the transfer membrane in the mixture described above. The color development of the immunoreactive bands on the transfer membrane was stopped by two successive 10 min. rinses with dH_2O and then air dried.

E. Determination of Protein Concentration

E.1 BioRad Protein Assay

Protein concentration was determined by the BioRad Protein Assay (TM) using the BioRad protein dye reagent (181) and bovine serum albumin (BSA; Sigma Chemical Co.) as standard. A one-hundred microliter aliquot of sample was added to a well of a 96-well round bottom microtiter plate (Flow Laboratories, Mississauga, Ontario). To this was added a 25 µl aliquot of BioRad protein assay dye reagent which was well mixed with the sample by pipetting. The absorbance of the mixture at 620 nm was measured spectrometrically using a Titertek Multiscan MC (mode 1, filter 7; Flow Laboratories). BSA samples of known concentrations were processed in an identical manner to that described above and their absorbances at 620 nm were determined. These values were plotted against their known concentration (expressed in µg/ml). The absorbance at 620 nm of the sample of unknown concentration was then compared with the absorbance values the BSA standards of known concentrations. From this the protein concentration of the sample of unknown concentration was determined.

E.2 Densitometric Analysis

The lanes of interest, including lanes containing different known concentrations of AFP, of an SDS-polyacrylamide gel were scanned by an LKB Laser Densitometer and the profile recorded by an Epson FX80 printer. The peaks of interest including controls were cut and weighed. This technique of cutting and weighing relevant peaks was utilized as the method of integration to determine the remaining undigested AFP and the proportion of the various cleavage products in the limited proteolytic digestions of AFP.

VI Limited Proteolytic Digestion of AFP

A. Micro-Scale Limited Proteolytic Digestion

A protocol was developed for the proteolytic digestion of mouse AFP using microgram amounts of the protein. It was necessary to determine which proteases and optimum conditions were required to obtain a limited proteolysis of AFP that would result in a mixture various-sized peptides with the least remaining undigested protein.

Proteolytic digestion of AFP with various individual proteases was performed in different buffer systems, with different protease to AFP ratios (referred throughout this thesis as enzyme:substrate [E:S] ratios [w/w]), incubated at 37^{o}C for different time periods. The different buffer systems differed in their chemical composition, concentration and/or pH. Each buffer system consisted of a particular buffer not containing any denaturing agent (non-denaturing buffer) and the same buffer containing 0.1% (w/v) SDS (denaturing buffer). The amount of AFP used in each of the proteolytic digestions performed in these studies was 50-100 µg. The incubation periods included for short timed incubations were 1 hr. or 2 hrs. with a single aliquot of protease added initially, i.e. immediately prior to incubation. Where required, a second equal aliquot of protease

was added at 30 mins. into the mixtures incubated for periods of 1 hr. or 2 hrs. Longer incubation periods consisted of a total incubation period of 4 hrs. or 10 hrs., with a single aliquot of protease added initially. Where required, a second equal aliquot of protease was added 2 hrs. into the mixtures incubated for periods of 4 hrs. or 10 hrs. Immediately after the incubation was completed, the protease-treated AFP solution was heated at 100°C for 20 mins. to inactivate the protease (182).

Controls for the buffer system employed for each protease consisted of (i) AFP alone (at 2 mg/ml), and (ii) protease alone (at the greatest concentration of protease present in the digests) each prepared separately in the denaturing buffer employed for that given protease. These were incubated at 37°C for 2 hrs. or 10 hrs. followed by heating at 100°C for 20 mins. Upon completion of the manipulations, all of the protease-treated AFP solutions, the AFP control, and protease controls were stored at -20°C until the next day, when they were analyzed by SDS-PAGE (182). This was carried out with a 15% (w/v) acrylamide slab SDS-gel (as described in Section IV, C.2).

B. Small-Scale Limited Proteolytic Digestion

Small-scale limited proteolytic digestion of mouse AFP was performed with 2 mg of the protein. This was carried out for each of the proteases studied in Table 1 under optimum conditions determined (see Table 3) by analysis of the results of studies of the micro-scale limited proteolytic digestion of AFP studies (described above).

Small-scale limited proteolytic digestion of AFP was carried out employing 2 mg of the protein (at 2 mg/ml) in the non-denaturing or the denaturing buffer of a given buffer system for each protease (see Table 3) at an E:S ratio of 1:10 (w/w), except in the case of endo Arg-C where an

E:S ratio of 1:25 (w/w). The proteolytic digestion was incubated at 37°C with the first aliquot of protease added initially and a second equal aliquot of protease was added 2 hrs. into the total incubation period of 4 hrs. Immediately after the total incubation period was completed, the protease-treated AFP solution was heated at 100°C for 20 mins. to stop proteolysis.

Controls included (i) AFP at 2 mg/ml prepared in PBS incubated at 37°C for 4 hrs. and heated at 100°C for 20 mins., and (ii) protease alone at the concentration of protease utilized in the digestion of AFP (in the non-denaturing or the denaturing buffer employed for that protease) and processed as was the AFP control described above. An aliquot of each protease-treated AFP solution, AFP control and protease control was analyzed by SDS-PAGE carried out using a 15% (w/v) acrylamide slab SDS gel, following dialyzation against PBS in tubing membrane of 3500 MW cut-off for 48 hrs., and the remaining solutions were stored at -20°C for further use.

C. Large-Scale Limited Proteolytic Digestion

Large-scale limited proteolytic digestion of mouse AFP consisted of dissolving 20-40 mg of the lyophilized protein in ddH_2O (at a concentration of 4 mg/ml or 40 mg/ml) and adding an equal volume of non-denaturing buffer at 2 times the final desired concentration. The mixture was incubated at 37° with an aliquot of protease (at 10 mg/ml) added initially and with a second equal aliquot of protease added 2 hrs. into the total incubation period of 4 hrs. The proteases and the corresponding non-denaturing buffer employed in the large-scale limited proteolytic digestion of AFP, were (i) elastase in 0.1 M NH_5CO_3 pH 8.5, or (ii) endoproteinase Glu-C (endo Glu-C) in 0.1 M NH_5CO_3 pH 7.8, respectively. In the case of large scale proteolysis of AFP with elastase, the E:S ratio used was 1:25 (w/w) while the E:S ratio used with endo Glu-C was 1:10 (w/w). Immediately after the

total incubation period was completed, the proteolytic digestion was heated at 100°C for 20 mins. to inactivate the protease (182). An aliquot of each proteolytic digestion was then analyzed by SDS-PAGE and the remaining solution was stored at -20°C for further use.

VII Isolation of AFP-Peptide Fragments Produced by Large-Scale Limited Proteolysis

A. Gel Filtration Chromatography of Elastase-Treated AFP on a Sephacryl S-200 Column

Gel filtration chromatography of elastase-treated AFP was performed on a Sephacryl S-200 (superfine, fractionation MW range of 5-250 kilodaltons [Kd]; Pharmacia Ltd.) column (1.6 x 90 cm). The column was calibrated with standards of blue dextran (2000 Kd), β-amylase (200 Kd), alcohol dehydrogenase (150 Kd), bovine serum albumin (67 Kd), ovalbumin (43 Kd), and chymotrypsinogen A (25 Kd).

Elastase-treated AFP, following large-scale limited proteolytic digestion of 30 mg of mouse AFP by elastase (as described in Section VI, C), was dialyzed against 0.23 M ammonium formate pH 3.0, concentrated down to 5 ml with a Diaflo Ultrafiltration YM-10 membrane (10 000 MW cut-off; Amicon Canada Ltd.) in an Amicon Ultrafiltration Cell, and was further concentrated to 3.3 ml with an immersible CX-10 concentrator (Millipore Corp.). The dialyzed and concentrated elastase-treated AFP was applied to the Sephacryl S-200 column and eluted at room temperature with 0.23 M ammonium formate pH 3.0 at a flow rate of 14.8 ml/hr. One and a half milliliter fractions were collected. The absorbance of every second fraction collected was monitored electrophoretically at 280 nm and the absorbance of every fraction in the peak area was then monitored at 280 nm. as well. In addition, the BioRad protein assay was employed to detect peptides, among collected fractions, that may not have been detectable by

measuring their absorbance at 280 nm. Fractions before and after peak area were pooled and concentrated by ultrafiltration on YM-10 membrane, further concentrated with immersible CX-10 concentrator and dialyzed against PBS in tubing membrane 3500 MW cut-off. Each of the individual fractions in the peak area were also dialyzed against PBS. After dialysis, 15 µl aliquots of each of the above fractions were analyzed by SDS- PAGE on a 15% (w/v) acrylamide mini slab gel.

B. Successive Zone Elution of Elastase-Treated AFP by Preparative SDS-Polyacrylamide Gel Disc Electrophoresis

Successive zone elution of elastase-treated AFP by preparative SDS-polyacrylamide gel disc electrophoresis was performed with a Shandon model SAE-2782 preparative disc electrophoresis apparatus (Shandon Scientifec Co., London, England). Disc electrophoresis was carried out according to the dissociating SDS-discontinuous buffer system of Laemmli (156) (as described in Section V, B). In a 4.5% (w/v) acrylamide stacking gel (3 cm high) and a 15% (w/v) acrylamide resolving gel (10 cm high) supported by a 7% (w/v) polyacrylamide gel column, separated from the resolving gel by a membrane (3500 MW cut-off).

Pre-electrophoresis was performed at 30 mA with a pump rate of 13.2 ml/hr. for 40 mins. Forty milligrams of elastase-treated AFP (as described in Section VI, C), in a total volume of 2 ml, was mixed with 1 ml of a solution consisting of 0.0625 M Tris-HCl pH 6.8, 20% (w/v) glycerol, 0.2 M DTT, and 0.1% (w/v) bromophenol blue. The protein sample was then heated at 100°C for 5 mins., allowed to cool, and then layered (using a syringe) onto the top of the stacking gel. Electrophoresis was carried out using a constant current of 30 mA through the stacking gel, and of 40 mA through the resolving gel, with increases of 10 mA every 15 mins. until the current was set at 60 mA. The pump rate was 13.2 ml/hr.

Two-milliliter fractions were collected over a period of 11 hrs. Every second fraction collected was monitored at 280 nm and the absorbance of every fraction in the peak area was then monitored at 280 nm as well. A twenty-microliter aliquot of every second collected fraction was analyzed on a 15% (w/v) acrylamide slab gel by SDS-PAGE and fractions were pooled accordingly, dialyzed against ddH_2O in a tubing membrane of 3500 MW cut-off, frozen at -70°C, and lyophilized. The lyophilized material was weighed on an analytical Mettler AE 163 balance (Mettler Instrument Corporation, Hightown, N.J.), resuspended with PBS to a concentration of 2 mg/ml of total protein and peptide, and stored at -20°C for use at a later date.

C. Reverse-Phase High Performance Liquid Chromatography of Endoproteinase Glu-C-Treated AFP

High performance liquid chromatography (HPLC) was performed at room temperature with a Waters HPLC system consisting of two Waters model 590 Programmable Solvent Delivery systems, a Waters 840 Data and Chromatography Control Station, a Waters Wisp™ 710B Intelligent Sample Processor with a 200 µl loop, and a Waters 490 Programmable Multiwavelength Detector which was used to spectrophotometrically monitor eluants at wavelengths of 206 nm and 280 nm. Solvents employed were HPLC grade and degased by bubbling helium through them for 15 mins. The following solvents were employed for reverse-phase HPLC: Solvent A (the starting solvent, used to equilibrate the column prior to each run) consisted of 5% (v/v) acetonitrile (Caledon Laboratories Ltd., Georgetown, Ontario) with 0.1% (v/v) trifluoroacetic acid (TFA; Pierce Chemical Company, Rockford, Illinois) in HPLC grade water (Caledon Laboratories Ltd.). Solvent B consisted of 60% (v/v) acetonitrile in HPLC grade water with 0.1% (v/v) TFA.

All samples were chromatographed at a flow-rate of 1 ml/min., unless indicated otherwise, and individual 1 ml fractions were collected. Each fraction was dried under vacuum, resuspended in PBS and a 2.5 µl aliquot of each fraction was analyzed using a 15% (w/v) acrylamide mini-slab gel and the dissociating SDS-discontinuous buffer system of Laemmli (156).

C.1 Analytical Chromatography

Preliminary studies on the separation of endoproteinase (endo) Glu-C-treated AFP (as described in Section VI, C) were performed on a 30 cm x 3.9 mm I.D. C_{18} µBondapak™ reverse-phase column with a Guard-Pak Precolumn (end capped; Waters Associates, Milford, MA). Native mouse AFP represented the control. Lyophilized AFP was prepared in PBS at 20 mg/ml, while the endo Glu-C-treated AFP (20 mg/ml) was prepared by diluting the buffer, 0.1 M NH_5CO_3 pH 8.5, employed for the proteolysis with PBS and reconcentrating the sample to its original volume with a Centricon-10 microconcentrator (Amicon Canada Ltd.) prior to chromatography. Amounts of 300 µg of AFP or endo Glu-C-treated AFP, in a volume of 15 µl at 20 mg/ml prepared in PBS was applied onto the C_{18} column, which had been previously equilibrated with starting solvent, solvent A. The gradient of solvent B, employed for both the elution of AFP, and endo Glu-C-treated AFP, went from 0% to 33% B in 25 mins., from 33% to 82% B in 10 mins., from 82% to 83% B in 10 mins. and from 83% to 100% in 15 mins. The dried residue of each of the individual 1 ml fractions collected were resuspended in 20 µl of PBS and a representative 2.5 µl aliquot of each fraction was analyzed by SDS-PAGE.

C.2 Preparative Chromatography

Preparative chromatography of endo Glu-C-treated AFP was performed with reverse-phase HPLC. Eight milligrams of the endo Glu-C-treated AFP

(prepared in PBS as described above), at 20 mg/ml in a total volume of 400 µl, was applied to a 30 cm x 3.9 mm I.D. C_{18} µBondapak™ reverse-phase column (Waters Associates) or to a 25 cm x 45 mm I.D. C_{18} Vydac reverse-phase column (Separations Group, CA). Each of the columns were fitted with a Guard-Pak Precolumn (Waters Associates).

Chromatography performed on the C_{18} µBondapak™ column was carried out with the same gradient of solvent B as described above. The dried residue of each of the individual 1 ml fractions collected from the C_{18} µBondapak™ column was resuspended in 40-100 µl of PBS and a representative 2.5 µl aliquot of each of these fractions was analyzed by SDS-PAGE as described above. Certain of these analyzed fractions were rechromatographed (30-50 µl) on the same C_{18} µBondapak™ column at a flow-rate of 1 ml/min. with a gradient of solvent B that went from 0% to 33% B in 5 mins., from 33% to 82% B in 10 mins., from 82% to 83% B in 10 mins, and from 83% to 100% B in 10 mins. One-milliliter fractions were again collected, dried under vacuum, resuspended with 20 µl of PBS and analyzed by SDS-PAGE.

Chromatography performed on the C_{18} Vydac column was carried out at a flow-rate of 3 ml/min. with the same gradient of solvent B as described above used in the rechormatography of certain fractions. The dried residue of each of the individual 1 ml fractions collected were also resuspended in 10 µl of PBS and analyzed by SDS-PAGE.

VIII Preparation of Synthetic AFP Peptides

A. Peptide Synthesis

Seven peptides were synthesized to correspond to particular peptide sequences of mouse AFP. These AFP synthetic peptides are denoted as AFP-1/18, AFP-34/48, AFP-105/124, AFP-320/339, AFP-372/384, AFP-451/462, and AFP-525/545 as are indicated in Fig. 26; the first number designates

the amino-terminal corresponding to the peptide sequence of native mouse AFP and the second number designates the carboxyl-terminal corresponding to that peptide sequence. Each of the synthetic peptides were synthesized by Merrifield solid-phase synthesis techniques (113) by using an Applied Biosystems 430A peptide synthesizer, desalted by gel filtration on a Sephadex G-50 (Pharmacia Ltd.) column, purified by reverse-phase HPLC on a 30 cm x 3.9 mm I.D. C_{18} μBondapak™ reverse-phase column, and their identities were verified by HPLC and amino acid analysis (113,183). All of the synthetic peptides were stored at -20°C until used at a later time.

B. Coupling of Synthetic AFP Peptides to Bovine Serum Albumin

Each of the synthetic AFP peptides were coupled to the protein carrier bovine serum albumin (BSA) with the coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDAC, Pierce Chemical Co.) and the following method (184). Forty milligrams of a synthetic AFP peptide, 40 mg of BSA and 40 mg of EDAC were dissolved in 40 ml of PBS. The reaction was permitted to proceed at 4°C for 14-15 hrs. The reaction mixture was then extensively dialyzed against PBS in a tubing membrane of 12 000 to 14 000 MW cut-off to remove any excess coupling reagent and any remaining uncoupled synthetic AFP peptide. The efficiency of coupling of the dialyzed material was estimated with the aid of HPLC and amino acid analysis. BSA alone that had been processed in a manner identical to that described above, and was designated *BSA, was used as the control. All of the BSA-coupled synthetic AFP peptides and *BSA were stored at -20°C for use at a later time.

IX Culture System and Functional Assay

1. Preparation of Lyt 1^+23^- Thymocyte Cells

Single cell suspensions were prepared from adult murine thymus by gentle mincing of the tissue through stainless steel mesh into PBS. The

cell mixture was mixed with a pasteur pipette and placed in 15 ml polystyrene tubes (Fisher Scientific Co.; Corning). Cells were washed twice in PBS by centrifugation at 1000 rpm in a Beckman TJ-6 centrifuge (Beckman Instruments Inc., Montreal, Quebec). The cells were then counted and assayed for viability by their ability to exclude 0.1% trypan blue. Only cell preparations with viabilities between 90-95% were utilized. Cytotoxic depletion of cells sensitive to the monoclonal antiserum (anti)Lyt 2.1 (NEN Canada, Lachine, Quebec) was performed in a two step negative selection protocol. Approximately 50-55 x 10^6 cells were treated with 0.6 mls of 1/1000 dilution of anti-Lyt 2.1 (previously determined optimal dilution) for 30 mins. at 37^{o}C in a water bath. The cells were then pelleted by centrifugation at 1000 rpm for 10 mins., antiserum removed, and the cells resuspended andincubated for 45 mins. at 37^{o}C in 1 ml of a 1/10 dilution of rabbit complement (Cedarlane Laboratories, Hornby, Ontario). Following incubation, treated cells were washed four times with PBS. Lyt $1^{+}23^{-}$ thymocyte cells were resuspended in RPMI 1640 medium (Johns Scientific, Toronto, Ontario) supplemented with 20 mM HEPES (Johns Scientific), 5 x 10^{-5} M 2-mercaptoethanol (Eastman, Rochester, New York), 4 mM 1-glutamine (Johns Scientific), 100 U/ml penicillin (Gibco, Montreal, Quebec), 100 U/ml streptomycin sulfate (Gibco), and containing 0.5% fresh NMS. Cells were counted and their concentration was adjusted.

Concanavalin-A Induced Proliferation Assay

Cultures were performed in 96-well round bottom microtiter plates (Flow Laboratories, Mississauga, Ontario). CBA/J Lyt$1^{+}23^{-}$ thymocyte cells were cultured to assay for reactivity to the mitogen concanavalin-A (Con-A) using a microculture system as previously outlined (186). Lyt $1^{+}23^{-}$ thymocyte cells (per well) were cultured in triplicate in 0.1 ml of RPMI

1640 supplemented as described above. A 50.0 µl aliquot of Con-A at 4 µg/ml (prepared in medium at 4 times the final desired concentration per well), or medium only was added. Samples to be tested for immunosuppressive activity in medium prepared at twice the final desired concentration per well was added at the start of the culture. The cell cultures were incubated in a humidified atmoshpere of 95% air and 5% CO_2 for 48 hrs. An aliquot of 1 µCi of ^{3}[H]TdR was added for the last 4 to 6 hrs. of culture. The cells were then harvested onto glass fibre mats (Flow Laboratories) using a Titertek Multiple sample harvester (Flow Laboratories). The insoluble radioactivity was counted using standard liquid scintillation procedures in a Beckman 8000 liquid scintillation counter (Beckman Instruments Inc., Montreal, Quebec). The arithmetic mean of the triplicate samples was determined and the results are expressed as counts per minute (cpm).

Results

I Purity Assessment of AFP Preparation

Mouse AFP was purified from mouse amniotic fluid by antibody-agarose affinity chromatography as described in Materials and Methods. Isolated AFP preparations (set at 2 mg/ml) from the fall-through of an anti-NMS affinity column, were assessed for purity by Ouchterlony immunodiffusion, immunoelectrophoresis, and by APAGE. The three major protein components of MAF are albumin, AFP and transferrin (67). Ouchterlony immunodiffusion did not reveal any contaminants in the preparations employed in this study. A single precipitin line was obtained with monospecific anti-mouse (Mo)AFP, but none occurred when tested with anti-NMS, anti-gamma, anti-Tf and anti-mouse ALB. Immunoelectrophoretic analysis (Fig. 1) confirmed the purity of the AFP preparation as demonstrated by the formation of a single precipitin arc with monospecific anti-MoAFP, but not with polyspecific anti-NMS. In addition, the homogeneity of the preparation was indicated by a single protein band shown with APAGE (Fig. 2).

II Analysis of Peptide Fragments of AFP Produced by Limited Proteolysis

A. Micro-Scale Limited Proteolytic Digestion of AFP - Analysis by SDS-PAGE for the Determination of Optimum Conditions

The degradative method chosen for the production of mouse AFP peptide fragments was limited proteolytic digestion. The first phase of the investigation required the selection of the protease(s) to be used and the optimum conditions for limited proteolysis of AFP. A protocol utilizing microgram amounts of AFP was developed taking into account the limited amount of pure mouse AFP available and various undetermined parameters (such as incubation period, buffer, pH, and enzyme:substrate ratio) for the limited proteolysis of AFP. Considering the amino acid sequence of mouse

AFP (83,100) and the specificity of various proteases (Table 1) a battery of enzymes and sets of digestion conditions were employed, as demonstrated in Table 2. The cleavage products and the efficiency of the degradation process under the various conditions utilized in Table 2 were analyzed by SDS-PAGE using a 15% (w/v) acrylamide resolving gel. The gel was stained with Coomassie Blue. Each experiment was repeated independently three times and the results were found to be reproducible. Representative results are demonstrated in Figures 3-8.

Digestion patterns produced from the proteolysis of AFP were seen to be unique for each of the proteases utilized. This was demonstrated by the AFP digestions performed using such proteases as endo Glu-C (Fig. 3), endo Lys-C (Fig. 4), endo Arg-C (Fig. 5), chymotrypsin A_4 (Fig. 6) and elastase (Fig. 7 and 8). Mouse AFP was cleaved to various degrees by almost all of the proteases listed in Table 2. The proteolytic digests, in general, were not free of all unidgested AFP. Exceptions were papain, pronase and thermolysin digests in non-denaturing and denaturing buffers, as well as bromelain and elastase digests (Fig. 7 and 8) in denaturing buffer. These demonstrated no remaining undigested protein. However, no apparent cleavage of AFP was observed with thermolysin in the pH 7.8 buffer system and none was seen with trypsin, nor endo Arg-C (Fig. 5) in non-denaturing buffer. Exposure of AFP to 37°C for different time periods as well as heating at 100°C for 20 mins. does not appear to have caused degradation of the molecule. This was demonstrated by the fact that there was no change in band pattern as depicted in Figures 3-8, lanes 1 and 2.

In general, for a given protease and set of conditions, the digestion pattern generated by the proteolytic digestion of AFP in non-denaturing buffer was different than that produced by the digestion performed in

denaturing buffer (as demonstrated in Figs. 4-8). For example, larger peptide fragments (apparent MWs of 50-62 Kd) and a wider size range of fragments (apparent MWs of 12-62 Kd) were observed in the chymotrypsin A_4 digestion of AFP performed in non-denaturing buffer as compared to digestions in denaturing buffer (generated cleavage products with an apparent MW range of 10-23 Kd) (Fig. 6). In addition, there was less remaining undigested AFP observed in denaturing buffer.

Variation in the E:S ratio for a given protease, keeping all other conditions constant, resulted in differences in the intensity of the protein band corresponding to remaining undigested AFP and/or in the intensity of some of the peptide bands corresponding to the larger peptide fragments. The intensity of the peptide band corresponding to a peptide fragment with an apparent MW of 43Kd was observed to have decreased in the endo Glu-C digestion of AFP performed in denaturing buffer as the concentration of protease was increased by 5-fold (Fig. 3, lane 3 and 11). Similarly, the intensity of the protein band corresponding to remaining undigested AFP of the elastase digestion performed in non-denaturing buffer was noted to have decreased as the protease concentration was increased by about 3-fold (Fig. 3, lane 3 and 11). In the endo Lys-C digestion of AFP in denaturing buffer, the intensity of the protein band corresponding to remaining undigested AFP and that of two peptide bands corresponding to cleavage products with apparent MWs of 40 and 43 Kd, was observed to decrease as the protease concentration was increased by 4-fold.

The pH of the buffer system utilized for a given protease was observed to be crucial to the digestion pattern. This was demonstrated by thermolysin digestions of AFP in buffers differing from each other only in pH (pH 7.8 versus pH 8.5). The buffers employed in these digestions were

within the reported functional pH range (pH 7.0-9.0) for thermolysin (139). AFP was not digested by thermolysin in the pH 7.8 buffer system. In contrast, AFP was cleaved by thermolysin in the pH 8.5 buffer system and resulted in proteolytic fragments of various sizes (apparent MWs ranging from 8-54 Kd).

Variation in the total incubation period (1, 2, 4 or 10 hrs.), while all other conditions were kept constant, had no significant effect on the peptide patterns or the intensity of the protein band corresponding to remaining undigested protein. This was demonstrated by the elastase digestions of AFP (see Fig. 7, lane 3 and 4, and Fig. 8, lanes 3 and 4).

B. Small-Scale Limited Proteolytic Digestion of AFP - Summary of Optimum Conditions Determined

Optimum conditions have been determined for the limited proteolytic digestion of AFP carried out in non-denaturing and denaturing buffers for each of the proteases utilized (listed in Table 1) and these are summarized in Table 3. These conditions were defined by analysis of the results of the micro-scale limited proteolytic digestion studies presented above in which each digestion was performed using 50-100 µg of AFP. The set of conditions which generated peptide fragments over a range of sizes with the least remaining undigested protein were considered as optimum. These conditions were therefore utilized in the small-scale proteolytic digestions of AFP (performed using 2 mg of AFP per digestion).

B.1 Physicochemical Characterization

The proteolytic fragments present in digests resulting from digestions carried out under the optimum conditions determined (summarized in Table 3) were physicochemically characterized by SDS-PAGE, IEF-PAGE and APAGE. The cleavage products were analyzed for size by SDS-PAGE using a 15% (w/v) acrylamide resolving gel and are depicted in Figures 9-12. The apparent

molecular weight range of the proteolytic fragments and the corresponding percentage (%) of undigested AFP remaining in each of the proteolytic digests are indicated in Table 3. The digestion patterns of the small-scale limited proteolytic digestions are similar to those observed for the micro-scale digestions performed under identical conditions. In general, it was observed that there were no significant differences in the peptide band patterns. However, the intensity of the protein band corresponding to remaining undigested AFP was observed to be greater in the small-scale digestions than in the micro-scale digestions. This was demonstrated by the elastase digestion of AFP in non-denaturing buffer (Fig. 8, lane 13) as compared to the micro-scale digestion performed under the same conditions (Fig. 10). The digestion patterns from the small-scale limited proteolytic digestion studies, when compared from one experiment to the next, were found to be reproducible, as was the case for the micro-scale limited proteolytic digestion studies.

Electrophoresis of the protease-treated AFP solutions by APAGE was performed using a 15% (w/v) acrylamide resolving gel. Each of the proteolytic digestions appeared as a smeared band which, in many cases, had migrated to the area where undigested AFP is observed to migrate. Therefore, there were no distinguishable differences in the electrophoretic migration of the proteolytic fragments of each of the protease-treated AFP solutions under alkaline conditions. Also, the isoelectric points of the proteolytic fragments of these protease-treated AFP solutions could not be determined with the aid of IEF-PAGE because of the poor resolution of the cleavage products by this technique. In some cases no bands were detected when the gel was stained with Coomassie Blue. Bands were detected in certain of the proteolytic digests, but these corresponded to undigested AFP. These particular proteolytic digests, when analyzed by SDS-PAGE, showed the presence of remaining undigested protein.

B.2 Immunological Characterization

Testing of the antigenicity of the proteolytic fragments of the digests of AFP was performed by Ouchterlony immunodiffusion, immunoelectrophoresis and immunoblotting using monospecific anti-MoAFP.

Ouchterlony immunodiffusion demonstrated a single precipitin line for those proteolytic digests that contained residual undigested AFP as determined by SDS-PAGE. There was no precipitin line observed with any of the protease controls corresponding to these proteolytic digests (protease control consists of a given protease which has been processed as decribed in Materials and Methods, Section B, page 36). Immunoelectrophoresis of the protease-treated AFP solutions was performed with monospecific anti-MoAFP. Single precipitin arcs were observed, in the same position relative to control AFP, in those proteolytic digests which contained residual undigested protein. An exception was observed in the endo Arg-C digestion performed in denaturing buffer. Immunoelectrophoresis of this digest generated a precipitin arc which extended to the right of the position (toward the anode) relative to control AFP. There was no reaction with any of the protease controls.

Immunoblotting is 10^3-fold more sensitive in the detection of antigen than is Ouchterlony immunodiffusion or immunoelectrophoresis (186). Analysis of the antigens by this technique, in combination with SDS-PAGE, which permits their separation based on molecular weight, allows individual components of a complex mixture of peptides to be immunologically characterized and distinguished.

The immunoblotting procedure was carried out for each of the proteolytic digests and their corresponding protease controls. The immunoblots are depicted in Figures 13B-15B and their corresponding SDS-polyacrylamide mini-slab gels, which were stained with Coomassie Blue, are shown in Figures 13A-15A. An immunoreactive band corresponding to

undigested AFP was detected by immunoblotting in proteolytic digests which were observed to contain residual undigested protein as determined by SDS-PAGE. However, in certain digests, only some of the actual number of peptide bands determined by SDS-PAGE analysis were detected by immunoblotting experiments. For example, SDS-PAGE analysis of the endo Arg-C digestion of AFP performed in denaturing buffer revealed 10 well-resolved peptide bands (Fig. 14A, lane 11), but only 4 related immunoreactive bands were demonstrated by immunoblotting Fig. 14B, lane 11). Also, the endo Arg-C digestion carried out in non-denaturing buffer was found to contain 4 resolved peptide bands as determined by SDS-PAGE (Fig. 14A, lane 10), but no immunoreactive bands were detected in the corresponding immunoblot (Fig. 14B, lane 10). In the case of the endo Lys-C digestion in denaturing buffer, 8 peptide bands were demonstrated by SDS-PAGE (Fig. 14A, lane 14). However, this digest was found to have only 4 immunoreactive bands as determined by immunoblotting experiments (Fig. 14B, lane 14). This may be interpreted to be due partly to the low concentrations of these particular peptide fragments. However, their band intensity in the SDS-PAGE as compared to the other protein bands in the proteolytic digest appears to be of the same intensity and thus can be considered to be present in about the same quantities. Therefore, there are digests in which some but not all of the cleavage products were observed to react with monospecific anti-MoAFP by immunoblotting and to retain some antigenic properties of the intact parent molecule.

III Isolation of AFP Peptide Fragments Produced by Large-Scale Limited Proteolysis

Scaling-up of particular small-scale limited proteolytic digestions of AFP were performed in order to obtain quantitative amounts of these digests. These large-scale limited proteolytic digestions of AFP

(performed with 20-40 mg of the protein) were utilized in separation studies attempting to isolate certain proteolytic fragments. The results of these studies are outined in this section.

A. Gel Filtration Chromatography of Elastase-Treated AFP on a Sephacryl S-200 Column

Elastase was one of the proteases utilized in the large-scale limited proteolytic digestions of AFP. This protease was chosen because of the cleavage products observed to be generated by the small-scale elastase digestion of AFP performed in non-denaturing buffer (see Fig. 10). SDS-PAGE analysis of this particular proteolytic digest indicated 8 well-resolved peptide fragments which differed greatly in apparent MWs (ranging from 12-59 Kd) and contained very little undigested AFP.

Large-scale limited proteolytic digestion of AFP with elastase was carried out with 30 mg of AFP, as described in Materials and Methods. This generated a mixture containing a few small peptide fragments with apparent MWs of 12-18 Kd, two large peptide fragments with apparent MWs of 50 and 59 Kd (major fragment comprising approximately 82% of the intact parent molecule) and remaining undigested protein as determined by SDS-PAGE (Fig. 16B). It has been reported that proteolytic digestion of a protein by a given protease for a given set of conditions when scaled-up can result in a different digestion pattern (139). Although the digestion pattern of the large-scale elastase digest is not identical to that of the small-scale digest, similarities do exist. A major peptide band corresponding to an apparent MW of 59 Kd present in the small-scale elastase digest (Fig. 10, lane 3) is also observed in the large-scale elastase digest (Fig. 16B, lane 3). Small peptide fragments with an apparent MW range of 12-18 Kd are also present in both the small-scale elastase digest (Fig. 10, lane 3) and in the large-scale elastase digest (Fig. 16B, lane 3; the peptide bands corresponding to apparent MWs of 12-18 Kd are very faint, but were observed

on the actual SDS-gel). Separation of smaller cleavage products from the larger peptide and from remaining undigested AFP in the large-scale proteolytic digest was attempted by conventional gel filtration chromatography with an alkaline non-dissociating buffer. However, resolution of the cleavage products and the remaining undigested AFP was not accomplished. Other attempts to separate these components were also made utilizing alkaline dissociating buffers which contained the denaturing reagents SDS or DTT, but resolution was still not achieved.

A low pH eluting buffer, 0.23 M ammonium formate pH 3.0, which has been used in one of several gel filtration chromatography steps employed in the isolation of fragments of BSA produced by limited proteolysis (134,147) was next utilized. Gel filtration chromatography of 30 mg of elastase-treated AFP on the Sephacryl S-200 column was performed with 0.23 M ammonium formate pH 3.0 as the eluting buffer (the elution profile is displayed in Fig. 16A). SDS-PAGE analysis of aliquots of fractions 57, 72 and 77 are depicted in Figure 16B, lanes 4-6, and represent the kind of separation that was obtained. Partial separation of cleavage products (MWs of 12-18 Kd) from larger peptide fragments (MWs of 50-59 Kd) and most of the remaining undigested AFP was observed (Fig. 16B, lane 6). However, co-chromatography of cleavage products and residual undigested protein was also noted (Fig. 16B, lane 5). The protein concentration of the collected fractions was determined (see Table 4) using the BioRad Protein Assay with BSA as standard. The recovery of total protein and peptide from the column was approximately 18.6 mg (62%). Fractions 57, 72 and 77 were tested for their antigenicity by Ouchterlony immunodiffusion as well as by immunoblotting, both of which were performed using monospecific anti-MoAFP. In Ouchterlony immunodiffusion, a similar precipitin line was observed with each of the fractions as was observed with the unfractionated elastase digestion. This might have been due to the presence of interfering traces

of undigested protein in the fractions. However, the cleavage products of MWs 18 and 59 Kd in fraction 72 analyzed by SDS-PAGE (Fig. 17A, lane 5) were observed to retain antigenic properties of the native protein as demonstrated by immunoblotting experiments (Fig. 17B, lane 5).

B. Successive Zone Elution of Elastase-Treated AFP by Preparative SDS-Polyacrylamide Gel Disc Electrophoresis

A large-scale limited proteolytic digestion of AFP by elastase was carried out with 40 mg of AFP, as outlined in Materials and Methods. Analysis of an aliquot of this digest by SDS-PAGE revealed a mixture of 10 peptide fragments with an apparent MW range of 14-59 Kd and a trace of undigested protein (Fig. 18B, lane 3). Successive zone elution of the elastase digestion of AFP was performed by preparative SDS-polyacrylamide gel disc electrophoresis. This technique was chosen to isolate the cleavage products of different molecular weights generated by the elastase digestion of AFP. Successful reproducible resolution of the cleavage products of the elastase digest was obtained by analytical SDS-PAGE. Therefore, the same acrylamide gel composition and buffer system utilized in the analytical SDS-PAGE were employed in the preparative SDS-polyacrylamide gel disc electrophoresis.

Two-milliliter fractions were collected from the polyacrylamide gel column once 40 mg of elastase-treated AFP had been applied onto the column. The elution profile of the successive zone elution of the elastase digestion demonstrated a single peak where the absorbance of each of the collected fractions was monitored at 280 nm (Fig. 18A). The SDS present in the buffer system may possibly have interfered with absorbance measurements. An aliquot (20 µl) of each of the individually collected fractions was analyzed by SDS-PAGE. This demonstrated that some of the cleavage products co-migrated. However, separation of peptide fragments with MWs of 14-22 Kd from fragments of 34-38 Kd was achieved, as was the

separation of these two sets of peptide fragments from fragments of 55-59 Kd and from residual undigested AFP (Fig. 18B). Therefore, partial separation of certain cleavage products was accomplished. However, none of the larger proteolytic fragments (apparent MWs 55-59 Kd) were found in the fractions collected from the polyacrylamide gel column. This was probably due to an insufficient period of electrophoresis which consequently did not allow larger fragments of slow mobility to migrate out of the column. The lengthy electrophoresis period required for the elution of high molecular weight peptides is a noted disadvantage of successive zone elution by preparative polyacrylamide gel disc electrophoresis.

Fractions analyzed by SDS-PAGE were then pooled according to their similar SDS gel patterns (see Table 5), dialyzed against ddH_2O, frozen at $-70^{o}C$ and lyophilized. The total protein and peptide recovery from the polyacrylamide column and following manipulations was 21%. The resuspended lyophilized samples 1-9 (see Table 5) were set at 2 mg/ml with PBS and an aliquot (10 µl) of each was analyzed by SDS-PAGE (Fig. 18B) which depicted the partial separation obtained from successive zone elution of elastase-treated AFP as described above. The antigenicity of the samples containing partially isolated cleavage products was investigated by immunoblotting using monospecific anti-MoAFP. Analysis of the SDS gel patterns of samples 1-9 and their pattern of immunoreactive bands demonstrated that only the proteolytic fragments of samples 2 and 3 (MWs of 12-14 Kd) (Fig. 19A and B, lanes 5 and 6, respectively) and of sample 6 (MW of 22 Kd) (Fig. 19A and B, lane 9) appear to have retained antigenic properties of the parent AFP molecule.

C. Reverse-Phase High Performance Liquid Chromatography of Endoproteinase Glu-C-Treated AFP

Endoproteinase (endo) Glu-C was utilized as the protease in scaling-up the limited proteolysis of AFP. This protease was selected beause of the

cleavage products which were observed to be generated by the small-scale endo Glu-C digestion of AFP carried out in non-denaturing buffer (see Fig. 9). A peptide mixture containing 15 fragments with apparent MWs ranging from 10-50 Kd and very little remaining undigested AFP was produced by the small-scale endo Glu-C digest. In addition, a greater number (approximately twice as many) and quantity of small peptide fragments were present in the endo Glu-C digest as compared to that found in the elastase digest of AFP (as observed by densitometric analysis of the Coomassie Blue stained SDS gels).

Large-scale limited proteolytic digestion of AFP by endo Glu-C (performed with 20 mg of AFP as outlined in Materials and Methods) yielded a complex mixture of peptide fragments (apparent MW range 10-50 Kd), of which a number of them were noted to be of low MWs (10-22 Kd). A technique which has been shown in many cases effective for the separation of peptide mixtures, in particular small peptide fragments, is reverse-phase HPLC. Therefore, this technique was utilized in the isolation of cleavage products generated by a large-scale endo Glu-C digestion of AFP. In addition, this technique was also favored because of its rapidity and the microgram quantities of protein required for a single run.

After performing many preliminary runs with different acetonitrile gradients containing 0.1% (v/v) TFA, analytical chromatography of the endo Glu-C digestion was carried out on a C_{18} μBondapak reverse-phase column as described in Materials and Methods. Two major peaks were observed by monitoring eluants spectrophorometrically at a wavelength of 206 nm (Fig. 20). Each of the one-milliliter fractions collected (from time 0 to the end of the 65 min. run) were frozen, lyophilized, and resuspended in PBS. SDS-PAGE analysis of an aliquot (2.5 μl) of each of the resuspended fractions revealed that the cleavage products observed in the endo Glu-C

digest had been eluted from the C_{18} μBondapak reverse-phase column. Also, there had occurred the separation of small peptide fragments (MW range of 10-22 Kd) from the other cleavage products (ranging in MWs from 26 to 50 Kd) and from remaining undigested AFP. These fragments, however, were also observed to have co-migrated. The protein recovery from the column was determined by having chromatographed 300 μg of mouse AFP (Fig. 21A) under identical conditions as the endo Glu-C digestion. The amount of protein recovered from the column was 210 μg (70%). The PBS-resuspended sample, consisting of the lyophilized pooled fractions from the entire run, was analyzed by SDS-PAGE (Fig. 21B).

Analytical chromatography of the endo Glu-C digest of AFP by reverse-phase HPLC allowed the partial separation of proteolytic fragments of low molecular sizes (10-22 Kd) from each other and from fragments of 26-50 Kd and residual undigested protein. However, co-elution of these fragments of different molecular sizes was also noted. Therefore, the next step taken involved the following: (i) applying the same conditions used in the analytical chromatography method to the separation of 8 mg of the endo Glu-C digest (preparative chromatography) on the same reverse-phase column (C_{18} μBondapak); and (ii) followed by rechromatography, where required, of individual fractions derived from the preparative chromatographic step.

The elution profile resulting from the preparative chromatography of endo Glu-C-treated AFP is shown in Figure 22A. SDS-PAGE analysis of the individual fractions revealed that the small cleavage products with apparent MWs of 10-22 Kd did separate from proteolytic fragments of MWs 26-50 Kd and from remaining undigested AFP as indicated by fractions eluted at 39 and 40 mins. (see Fig. 22B, lanes 4 and 5, respectively) of the preparative chromatographic step. However, these fragments also were observed to have co-chromatographed during the preparative run as

demonstrated by fractions eluted at 42, 43 and 44 mins. (Fig. 22B, lanes 7-9, respectively). There appears to be in the fraction from the preparative chromatographic step which eluted at 43 mins. (Fig. 22B, lane 8) non-distinct bands in the region in which AFP migrates as compared to a faint distinct band in the starting material (a 10-fold dilution of the actual concentration of the digest injected onto the column). This may be due to renaturation of the protein and/or aggregation of peptide fragments which may have occurred during the lyophilization process (132). The lyophilized material was resuspended in a small volume of PBS which is not a denaturing buffer and may not have permitted complete solubilization of the protein and/or peptide fragments. Consequently, this may have contributed to the poor migration of the fragments as observed in Figure 22B, lane 8. Some of the fragments may have migrated as a complex of peptides or renatured protein leading to unresolved components causing smearing of the lagging (top) portion of the pattern (depicted in Fig. 22B, lane 8) and region to which AFP migrates. The probable problem of renaturation and/or aggregation may be minimized if the lyophilized material were to be resuspended in a denaturing (5-10% SDS-containing) buffer instead of the non-denaturing buffer utilized (PBS). The peptide fragments in the collected fractions (related to any of the reverse-phase HPLC runs) cannot be compared quantitatively to one another nor to the endo Glu-C digestion of AFP because their concentrations are not relative with respect to each other. Also observed was the co-elution of fragments with apparent MWs of 43-50 Kd and remaining undigested proteins; small sized cleavage products with MWs ranging from 10-15 Kd were not present. This is depicted by fractions eluted at 45, 46 and 47 mins. (see Fig. 22B, lanes 10-12, respectively) of the preparative chromatographic step. The fraction eluted at 41 mins. during the preparative chromatographic run demonstrated

co-elution of peptide fragments with apparent MWs of 36 and 38 Kd, but observed to be free of all other fragments and remaining undigested AFP (Fig. 22B, lane 6). These two proteolytic fragments were noted to migrate in an SDS gel to an area proximal to the region where the protease itself is seen to migrate. The individual fractions from the preparative chromatographic step which eluted at 41 and 43 mins. and which were analyzed by SDS-PAGE were independently rechromatographed (Fig. 23A and 24A, respectively). Rechromatography was performed using a similar acetonitrile gradient containing 0.1% TFA. Individual one-milliliter fractions were collected at 1 min. intervals throughout the entire 40 min. run. Rechromatography of the fraction from the preparative chromatographic step eluted at 41 mins. did not result in the separation of the cleavage products as demonstrated (Fig. 23B) by SDS- PAGE analysis of the fractions 28 and 29 mins. of the rechromatographic step (Fig. 23A). However, rechromatography (Fig. 24A) of the fraction which eluted at 43 mins. of the preparative chromatographic step did permit the separation of the cleavage products from remaining undigested AFP. This was demonstrated by SDS-PAGE analysis of the fraction from the rechromatographic run which eluted at 30 mins. which revealed the absence of a protein band corresponding to undigested AFP (Fig. 24B). The latter may still have been bound to the column for it did not appear in any of the collected fractions, as determined by SDS-PAGE. Low molecular weight peptide fragments of approximately 10-13 Kd were observed in the fraction which eluted at 30 mins. from the rechromatography step. These peptide fragments were not present in the starting material. These additional small peptides were probably residual material which had remained bound to the column from the preparative chromatographic step and were displaced by other peptides or undigested protein only during the rechromatographic run.

As expected, for a small-pore support, the C_{18} μBondapak column is not very effective in separating all of the largest, most hydrophobic peptides (168) present in the endo Glu-C digest. This is demonstrated by the presence of remaining large proteolytic fragments of apparent MWs 40-50 Kd in the fraction which eluted at 30 mins. (Fig. 24B, lane 7) during the run to rechromatograph the fraction which was eluted at 43 mins. of the preparative chromatographic step. Preparative chromatography of endo Glu-C-treated AFP was also performed (as described in Materials and Methods) on a semi-preparative C_{18} Vydac reverse-phase column (Fig. 25A), using an acetonitrile gradient containing 0.1% TFA, over a 40 min. time period. Individual one-milliliter fractions were collected at 33 sec. intervals during the entire run. The individual fractions were analyzed by SDS-PAGE and the fractions which eluted between 17 mins. and 40 sec. and 19 mins., with a 20 sec. interval per fraction, (demonstrated in Fig. 25B, lanes 4-8), indicated the separation of small cleavage products with MWs ranging from 10-18 Kd from fragments with MWs of 26-50 Kd had occurred. Also, the co-elution of these fragments and remaining undigested AFP did occur (Fig. 25B and 25C, lanes 8-13 and lanes 4-8, respectively).

IV Synthetic AFP Peptides and Coupled Synthetic AFP Peptides - for Functional Analysis

1. Physicochemical Characterization.

All of the synthetic AFP peptides which were synthesized are depicted in Figure 26 (AFP-1/18, AFP-34/48, AFP-105/124, AFP-320/339, AFP-372/384, AFP-451/462 and AFP-525/545) and were found to be >95% pure by analytical HPLC. Their amino acid composition, calculated molecular weight, and solubility and pH in PBS are summarized in Table 7.

Biological activity has been observed with a synthetic 17-amino acid peptide (CKS-17) when it is coupled to the carrier protein BSA (119). This

is thought to be dependent upon a particuar conformation of the peptide molecule that is conferred upon it by coupling the peptide molecule to the carrier (119). Therefore, coupling synthetic AFP peptides to a carrier may permit them to fold into certain conformation(s) which might render the peptides biologically active. For this reason, the synthetic AFP peptides were coupled to BSA. The synthetic AFP peptides which underwent the coupling procedure were analyzed by amino acid analysis to determine if coupling had indeed occurred. Coupling of the peptides to BSA did occur. The efficiency of coupling was estimated, based on analysis of the amino acid results, for each of the peptides except AFP-320/339 (analysis of results obtained were inconclusive) (see Table 7 which also includes the concentrations of synthetic peptides coupled to BSA). The efficiency of coupling reported for CKS-17 to BSA was 30%, whereas that obtained of the synthetic AFP peptides to BSA ranged from 1.8-19.7%.

2. Immunological Characterization.

The synthetic AFP peptides did not react with monospecific anti-MoAFP as determined by Ouchterlony immunodiffusion or by immunoelectrophoresis. Testing the antigenicity of the synthetic peptides to monospecific anti-MoAFP by immunoblotting was inconclusive because of the poor resolution of these peptides on SDS gels. The BSA-coupled synthetic AFP peptides did not react with monospecific anti-MoAFP as observed by immunoblotting and immunoelectrophoresis. This may be explained by the low (μg/ml) concentration of peptide coupled to BSA or by the hypothesis that the synthetic peptides do not resemble any antigenic sites of the parent mouse AFP molecule. The coupled peptides did react with polyclonal anti-BSA in immunoelectrophoresis experiments. It may be that polyclonal anti-BSA still recognizes the BSA portion of the conjugated material, or that it may be reacting with the remaining uncoupled BSA.

3. Effect of Synthetic AFP Peptides Uncoupled and Coupled to BSA on the Lyt 1^+ 23^- Con-A Stimulated Thymocyte Assay.

Preliminary functional experiments were performed with the synthetic AFP peptides that were soluble in PBS. These were AFP-1/18, AFP-34/48 and AFP-525/545 which were tested for their effect on the Con-A response of Lyt 1^+ 23^- thymocyte cells. The peptide, AFP-34/48, corresponds to an amino acid sequence (amino acids 34-48) near the N-terminus of mouse AFP in Domain I, which has the least homology to human albumin). Among the synthetic peptides tested, AFP-34/48 demonstrated an anti-proliferative capacity at approximately an 8-fold greater concentration than that required of mouse AFP (see Table 8). AFP-34/48 had no significant effect on cultures to which no Con-A was added. In addition, this synthetic AFP peptide as well as the above-mentionned two soluble synthetic peptides did not affect cell viability as measured by the ability of cells to exclude trypan blue. None of the coupled synthetic AFP peptides (AFP-1/18-BSA, AFP-34/48-BSA and AFP-525/545-BSA) caused the suppression of the Con-A response of Lyt 1^+ 23^- thymocyte cells as depicted in Table 9.

Note: The results shown in Table 8 are those of a single experiment performed. Therefore, such an experiment should be repeated in order (i) to determine whether or not the results demonstrated in Table 8 are reproducible, and (ii) to permit comparisons of the results obtained from these experiments.

TABLES AND FIGURES

Table 1

Properties of proteases utilized in studies of the micro-scale limited proteolytic digestion of AFP. Shown are each of the types of proteases utilized in the proteolysis of mouse AFP (classified on the basis of their mechanism of action) as well as their specificity and optimum functional pH range for proteolytic action.

Table 1 **Properties of Proteases Utilized in the Micro-Scale Limited Proteolytic Digestion of AFP Studies**

Protease	[a]Type of Protease	[a]Specificity	[a]pH Optimum
Endoproteinase Glu-C from Staphylococcus aureus V8 MW:27000	Serine protease	Peptide bonds at the carboxylic end of Glu & Asp. Peptide bonds at the carboxylic end of Glu.	e.g. $NaHPO_4$, pH 7.8 e.g. NH_5CO_3, pH 7.8
Endoproteinase Lys-C from Lysobacter enzymogenes MW:30000	Serine protease	Amide or ester bonds and peptides and proteins at the carboxylic end of Lys only.	8.5 - 8.8
Endoproteinase Arg-C from mouse submaxillary gland MW:30000	Serine protease	Peptide and ester bonds at the carboxylic end of Arg.	8.0 - 8.5
Chymotrypsin A_4 from bovine pancreas MW:25000	Serine protease endopro-teinase	Peptide bonds involving aromatic L-amino acids (Tyr, Phe, Trp) and esters of susceptible amino acids. Cleavage of leucyl (Met, Asp) and glutamyl bonds.	7.0 - 9.0
Elastase from porcine pancreas MW:25900	Serine protease	Peptide bonds at the carboxyl end of amino acids bearing uncharged non-aromatic side chains (Ala, Val, Leu, Ile, Gly, Ser).	8.8

Thermolysin from *Bacillus thermoproteolyticus*	Metallo-protease, endoprotease	Peptide bonds (at the N-terminal) involving the amino group of hydrophobic amino acid residues with bulky side chains (Ile, Leu, Val, Phe, Met and Ala). To a minor extent, the amino sites of Tyr, Gly, Thr and Ser are also susceptible in some cases.	7.0 - 9.0
Trypsin from bovine pancreas MW:23500	Serine protease	Amino acid esters and amides at the carbonyl group of Arg & Lys.	8.0
Papain from Carica papaya	Thiol-protease	Amides and esters of amino acids and peptides, especially at bonds involving the amino acids Arg, Lys, Glu, His, Gly and Tyr.	6.0 - 7.0
Bromelain from Ananas comosus MW:33000	Thiol-protease	Non-specifically hydrolyzes proteins, amides and esters of amino acids and peptides.	5.0 - 7.0
Pronase from *Streptomyces griseus*	Mix of several proteases	Unspecific endogenous and exogenous cleavage of proteins.	6.0 - 7.5

[a] see Reference 139

Table 2

Conditions utilized in the studies of micro-scale limited proteolytic digestion of AFP. The micro-scale proteolytic digestions of mouse AFP were performed as described in Materials and Methods in non-denaturing and denaturing buffers. The proteases and sets of conditions utilized in these studies are indicated.

Table 2 Conditions Utilized in the Micro-Scale

Limited Proteolytic Digestion of AFP

Protease	Buffer Condition ND*	Buffer Condition D**	E:S Ratio (w/w)	Total Incubation Period (hrs)	Temperature of Incubation (°C)	Figure Number
Endoproteinase Glu-C	0.1 M NH_5CO_3, pH 7.8		1:50 1:25 1:10	4 & 10	37	--
		0.1 M NH_5CO_3, 0.1% SDS, pH 7.8	1:50 1:25 1:10	4 & 10	37	3
Endoproteinase Lys-C	0.1 M NH_5CO_3, pH 8.5	vs 0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:100 1:25	4 & 10	37	4
Endoproteinase Arg-C	0.1 M NH_5CO_3, pH 8.5	vs 0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:25 1:10	4 & 10	37	5
Chymotrypsin A_4	0.1 M NH_5CO_3, pH 8.5	vs 0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:33 1:10	4 & 10	37	6
	0.1 M NH_5CO_3, pH 8.5	vs 0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:33 1:10	1 & 2	37	--

Elastase	0.1 M NH_5CO_3, pH 8.5 **vs**	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:25 1:10	4 & 10	37	7
	0.1 M NH_5CO_3, pH 8.5 **vs**	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:25 1:10	1 & 2	37	8
Thermolysin	0.1 M NH_5CO_3, pH 7.8 **vs**	0.1 M NH_5CO_3, 0.1% SDS, pH 7.8	1:40 1:10	4 & 10	37	--
	0.1 M NH_5CO_3, pH 8.5 **vs**	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:40 1:10	4 & 10	37	--
Trypsin	0.1 M NH_5CO_3, pH 7.8 **vs**	0.1 M NH_5CO_3, 0.1% SDS, pH 7.8	1:100 1:10	4 & 10	37	--
Papain	0.125 M Tris, pH 6.8 **vs**	0.125 M Tris, 0.1% SDS, pH 6.8	1:50 1:10	4 & 10	37	--
Bromelain	0.125 M Tris, pH 6.8 **vs**	0.125 M Tris, 0.1% SDS, pH 6.8	1:50 1:10	4 & 10	37	--
Pronase	0.125 M Tris, pH 6.8 **vs**	0.125 M Tris, 0.1% SDS, pH 6.8	1:50 1:10	4 & 10	37	--

* Non-Denaturing
** Denaturing (i.e. non-denaturing buffer containing 0.1% SDS)

Table 3

Summary of optimum conditions determined for limited proteolytic digestions of AFP carried out in non-denaturing and denaturing buffers. The optimum conditions were determined from the analysis of the results of studies of the micro-scale limited proteolytic digestion of AFP. Each of the AFP digests were incubated at 37°C for a total period of 4 hrs. with an aliquot of protease added initially. The E:S ratio (w/w) for each protease and buffer system was as indicated, with a second equal aliquot of protease added at 2 hrs. The percentage (%) of remaining undigested AFP in each of the proteolytic digests, carried out under the indicated conditions, is shown and was determined by densitometric analysis. The apparent molecular weight (MW) range (expressed in kilodaltons [Kd]) of the proteolytic fragments present in each of the digestions was determined by SDS-PAGE and is indicated in brackets.

Table 3 **Summary of Optimum Conditions Determined for the Limited Proteolytic Digestions of AFP Carried Out in Non-Denaturing and Denaturing Buffers**

Protease	Buffer *ND	Buffer **D	E:S Ratio (w/w)	Apparent MW Range (Kd) of Proteolytic Fragments in Buffer *ND	Apparent MW Range (Kd) of Proteolytic Fragments in Buffer **D
Endoproteinase Glu-C	0.1 M NH_5CO_3, pH 7.8	0.1 M NH_5CO_3, 0.1% SDS, pH 7.8	1:10	10-50 (3.4)	8-50 (0)
Papain	0.125 M Tris, pH 6.8	0.125 M Tris, 0.1% SDS, pH 6.8	1:10	14-62 (5/4)	12-54 (0)
Trypsin	0.1 M NH_5CO_3, pH 7.8	0.1 M NH_5CO_3, 0.1% SDS, pH 7.8	1:10	— (100)	10-59 (0.8)
Chymotrypsin A_4	0.1 M NH_5CO_3, pH 8.5	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:10	14-59 (95.1)	10-59 (1.6)
Thermolysin	0.1 M NH_5CO_3, pH 8.5	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:10	10-54 (0.6)	10-54 (0)
Elastase	0.1 M NH_5CO_3, pH 8.5	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:10	12-59 (19.9)	10-37 (0)
Endoproteinase Arg-C	0.1 M NH_5CO_3, pH 8.5	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:10	— (—)	12-55 (30.0)
Endoproteinase Lys-C	0.1 M NH_5CO_3, pH 8.5	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5	1:25	— (—)	8-52 (7.0)
Bromelain	0.125 M Tris, pH 6.8	0.125 M Tris, 0.1% SDS, pH 6.8	1:10	12-50 (23.4)	10-52 (1.3)
Pronase	0.125 M Tris, pH 6.8	0.125 M Tris, 0.1% SDS, pH 6.8	1:10	15-50 (0)	— (0)

* Non-Denaturing ** Denaturing (i.e. non-denaturing buffer containing 0.1% SDS)
( ) Percentage of undigested AFP remaining in the proteolytic digest

Table 4

Determination of total protein and peptide concentration in fractions obtained from gel filtration of elastase-treated AFP on a Sephacryl S-200 column. The total protein and peptide concentration of each of the fractions was determined by the BioRad Protein Assay with BSA as standard.

Table 4

Determination of Total Protein and Peptide Concentration in Fractions Obtained from Gel Filtration of Elastase-Treated AFP on a Sephacryl S-200 Column.

Fraction Number	Total Protein and Peptide Concentration (μg/ml)
1 - 56	0
57	689.8
58	993.9
59	1096.4
60	1112.7
61	1152.4
62	1063.8
63	800.6
64, 65	814.5
66	609.5
67	581.6
68	614.4
69	215.8
70	330.0
71	350.9
72	207.2
73	316.0
74, 75	390.0
76	335.7
77	360.3
78	362.6
79 - 188	0

The percent recovery of protein and peptide: about 62%

Table 5

Weight of lyophilized peptide samples recovered from the successive zone elution of elastase-treated AFP by preparative SDS-polyacrylamide gel disc electrophoresis. Fractions were pooled based on the analysis by SDS-PAGE on a 15% (w/v) acrylamide gel of a 15.0 µl aliquot of every second 2.0 ml fraction collected. The pooled fractions were dialyzed against ddH_2O, frozen at $-70^{\circ}C$ and lyophilized. The lyophilized samples were weighed on an analytical balance.

Table 5

Weight of Lyophilized Peptide Samples Recovered from Successive Zone Elution of Elastase-Treated AFP by Preparative SDS-Polyacrylamide Gel Disc Electrophoresis

Fraction Number	Sample	Weight of Lyophilized Sample (mg)
1 - 27	1	1.65
28, 29	2	0.57
30, 31	3	0.51
32, 33	4	0.33
34, 35	5	0.40
36 - 39	6	0.60
40 - 49	7	1.39
50 - 55	8	0.92
56 - 72	9	1.98

Total weight of lyophilized protein and peptides: 8.35 mg.

The percent recovery of lyophilized protein and peptides: about 21%.

Table 6

Summary of physicochemical properties of the synthetic AFP peptides. The calculated molecular weight of each of the synthetic peptides was determined on the basis of their amino acid composition as indicated. The solubility of these peptides in PBS was designated according to visual observation.

Table 6 **Summary of Physicochemical Properties of Synthetic AFP Peptides**

		Synthetic Peptide						
		AFP-1/18	AFP-34/48	AFP-105/124	AFP-320/339	AFP-372/384	AFP-451/462	AFP-525/545
Amino Acid Composition (no. of residues)	Asp	1				1	1	
	Asn	1	1			1		1
	Thr	1	2	1	1			1
	Ser	3		1	4			
	Glu	2	4	2	3	4	1	4
	Gln	1	1	1		2		3
	Gly	1					3	
	Ala	2	1	4	1		1	
	Tyr				1			
	Val		2	1				1
	Met		1		2		1	1
	Ile	1			1	1	2	1
	Leu	2			1	2	1	5
	Phe	1	1	2	3		1	
	His	1			1	1	1	
	Lys	1	1	2	1	1		3
	Arg			1	1			
	Pro		1	5				1
Total no. of Amino Acid Residues		18	15	20	20	13	12	21
Calculated Molecular Weight (g/mole)		2251	2002	2508	2780	1839	1456	2882
[a]Solubility in PBS		IS	S	S	IS	IS	VIS	S
[b]pH in PBS		6	5 - 6	5 - 6	3	6	6	6 - 7

[a]Observed visually [b]Measured with litmus paper

IS = insoluble S = soluble VIS = viscous & insoluble

Table 7

Determination of the concentration of synthetic AFP peptide coupled to BSA and the efficiency of coupling. Each of the synthetic peptides was coupled to BSA and this material was analyzed by amino acid analysis to determine if coupling had occurred. Amino acid analysis also aided the estimation of the concentration of the synthetic peptides coupled to BSA and the efficiency of coupling. The final concentration of BSA in the solutions of conjugated material was 0.015 mM.

Table 7

Determination of the Concentration of Synthetic AFP Peptide Coupled to BSA and the Efficiency of Coupling

Synthetic Peptide Coupled to BSA	Concentration of Synthetic Peptide Coupled to BSA (μg/ml)	Efficiency of Coupling (%)
AFP-1/18	189.0	19.7
AFP-34/48	128.0	13.4
AFP-105/124	176.0	17.8
AFP-320/339	INC	INC
AFP-372/384	14.0	1.8
AFP-451/462	53.0	6.0
AFP-525/545	143.0	16.3

INC = inconclusive

Table 8

Effect of uncoupled synthetic AFP peptides on the Con-A response of Lyt 1^+23^- thymocyte cells. 250 000 Lyt 1^+23^- thymocyte cells were cultured for 48 hrs. in the presence of Con-A (1μg/ml) and various final concentrations of synthetic AFP peptides and AFP, as indicated. Background values are shown under the column designated "**Absence of Con-A**". The Lyt 1^+23^- cells were obtained by anti-Lyt 2.1 plus complement treatment of thymocyte cell preparations derived from adult CBA/J mouse thymuses. DNA synthesis was measured by the incorporation of [^{3}H]-TdR and is expressed as the mean counts per minute (cpm) $\pm$ standard error of the mean (S.E.M.) of triplicate cultures of one experiment. Preparation of cells, cultures and assay of proliferation were performed as described in Materials and Methods.

Table 8

Effect of Synthetic AFP Peptides on the Con-A Response of Lyt $1^{+}23^{-}$ Thymocyte Cells

Preparation	Conc. (μg/ml)	[^{3}H]-TdR incorporation mean cpm ± S.E.M.	
		Absence of Con-A	Presence of Con-A
Control (media)		187 ± 42	221 070 ± 4 733
AFP	200	363 ± 129	16 849 ± 1 113
	100	—	32 211 ± 3 835
	50	—	85 446 ± 9 403
	25	—	142 698 ± 8 193
AFP-34/48	400	—	72 191 ± 967
	300	—	100 455 ± 7 259
	200	—	155 714 ± 7 015
	100	—	181 749 ± 4 210
	50	—	239 575 ± 3 744
AFP-105/124	400	—	195 808 ± 10 729
	300	—	198 377 ± 10 547
	200	—	246 100 ± 19 348
	100	—	240 935 ± 6 382
	50	—	267 505 ± 6 497
AFP-525/545	400	—	185 989 ± 7 257
	300	—	215 866 ± 9 593
	200	—	230 750 ± 4 445
	100	—	266 930 ± 5 491
	50	—	246 655 ± 6 406

Table 9

Effect of synthetic AFP peptides coupled to BSA on the Con-A response of Lyt 1^+ 23^- thymocyte cells. 250 000 Lyt 1^+23^- thymocyte cells were cultured for 48 hrs. in the presence of Con-A (1 µg/ml) and various final concentrations of coupled synthetic AFP peptides, BSA, *BSA and AFP as indicated. The total concentration (uncoupled and coupled) of BSA in the solutions containing the conjugated material is shown in brackets. DNA synthesis was measured by the incorporation of [^{3}H]-TdR and is expressed as the mean counts per minute (cpm) $\pm$ S.E.M. of triplicate cultures of one experiment. Background values are shown under the column designated "**Absence of Con-A**". Cell preparations, cultures and assay of proliferation were performed as outlined in the legend of Table 8. *BSA, BSA alone that has undergone the same coupling conditions as used for the synthetic AFP peptides.

Table 9

Effect of Coupled Synthetic AFP Peptides to BSA on the Con-A Response of Lyt $1^{+}23^{-}$ Thymocyte Cells

Preparation	Conc. (μg/ml)	[^{3}H]-TdR incorporation mean cpm ± S.E.M.	
		Absence of Con-A	Presence of Con-A
Control (media)		1 853 ± 107	97 601 ± 5 804
AFP	200	257 ± 38	—
	50	—	24 921 ± 1 128
	25	—	41 415 ± 544
	12.5	—	53 135 ± 8 220
	6.25	—	48 005 ± 2 083
BSA	200	251 ± 38	—
	50	—	134 606 ± 8 528
	25	—	131 840 ± 6 779
	12.5	—	118 311 ± 5 058
	6.25	—	123 337 ± 4 624
*BSA	200	341 ± 100	—
	50	—	95 531 ± 10 408
	25	—	119 323 ± 8 275
	12.5	—	123 580 ± 8 188
	6.25	—	106 849 ± 10 783
AFP-34/48-BSA	25.6 (200)	465 ± 152	—
	6.4 (50)	—	109 009 ± 9 603
	3.2 (25)	—	102 931 ± 6 858
	1.6 (12.5)	—	106 236 ± 8 517
	0.8 (6.25)	—	98 276 ± 8 342
AFP-105/124-BSA	35.2 (200)	247 ± 44	—
	8.8 (50)	—	123 179 ± 9 874
	4.4 (25)	—	152 103 ± 9 755
	2.2 (12.5)	—	155 332 ± 6 188
	1.1 (6.25)	—	131 764 ± 26 398
AFP-525/545-BSA	28.6 (200)	275 ± 29	—
	7.15 (50)	—	115 319 ± 2 568
	3.58 (25)	—	141 865 ± 8 605
	1.79 (12.5)	—	110 747 ± 25 869
	0.89 (6.25)	—	108 252 ± 7 534

Figure 1.

Immunoelectrophoretic analysis of isolated mouse AFP preparation. Immunoelectrophoresis of a AFP preparation from the fall-through of anti-NMS affinity column was performed as described in Materials and Methods.

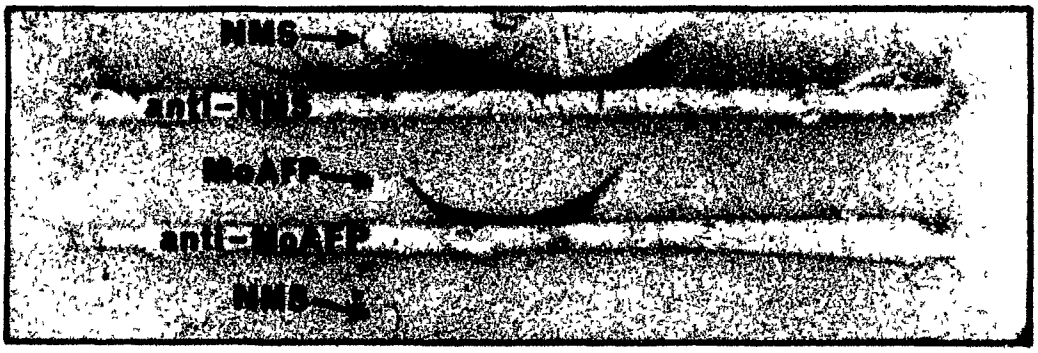

NMS
anti-NMS
MoAFP
anti-MoAFP
NMS

Figure 2.

Alkaline polyacrylamide gel electrophoresis analysis of isolated mouse AFP preparation. APAGE of mouse amniotic fluid (MAF), consisting of three major protein components (top band: albumin; middle band: AFP; bottom band: transferrin), and of the isolated mouse (Mo) AFP preparation shown in Fig. 1. The APAGE was performed using a 15% (w/v) acrylamide resolving gel. The gel was stained with Coomassie Blue.

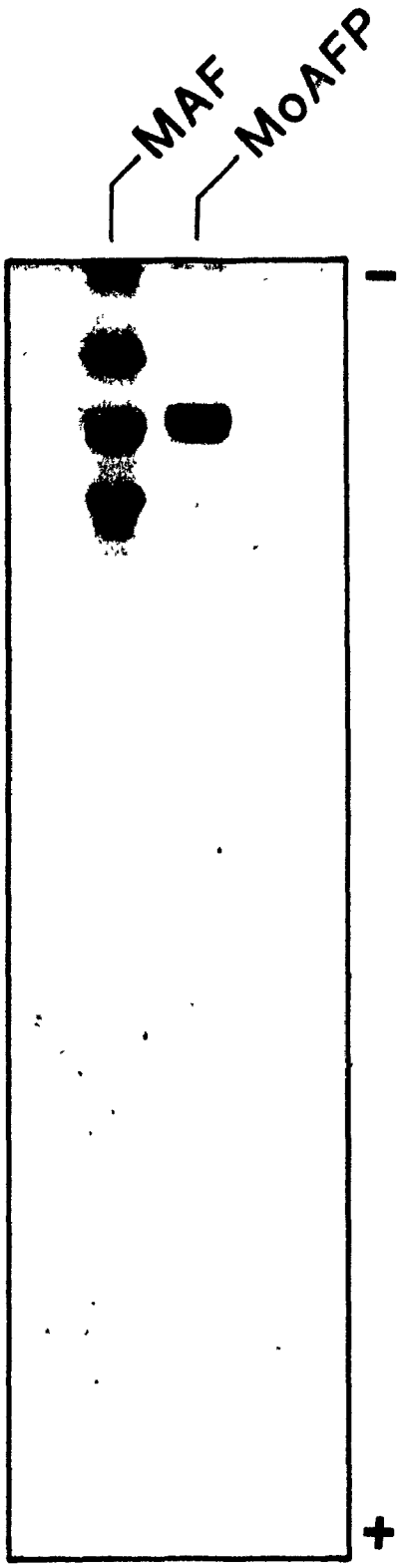
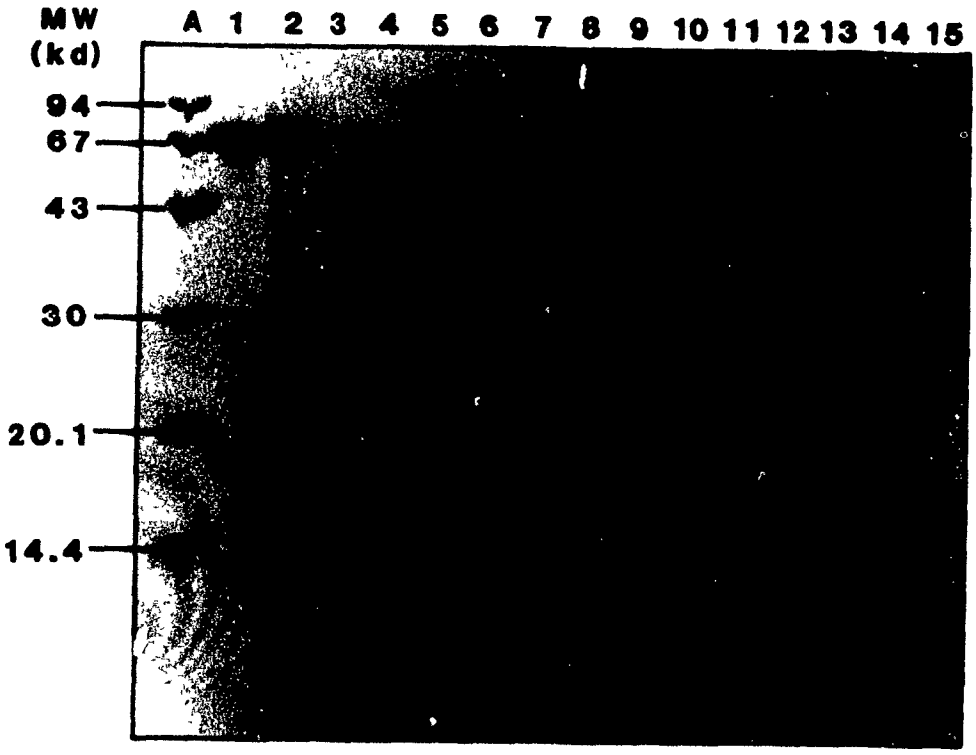

MAF
MoAFP
−
+

Figure 3.

Endoproteinase (endo) Glu-C proteolysis of AFP in denaturing buffer. SDS-PAGE of endo Glu-C-treated AFP and controls. Lane 1, AFP. Lane 2, AFP incubated at 37°C for 10 hrs. and followed by heating at 100°C for 20 mins. Lanes 3-14, endo Glu-C digestions of AFP carried out in 0.1 M NH_5CO_3 pH 7.8 containing 0.1% SDS (denaturing buffer) at an E:S ratio of 1:50 (lanes 3-6), 1:25 (lanes 7-10), or 1:10 (lanes 11-14). Lanes 3, 7 and 11, proteolytic digestions performed at 37°C for 4 hrs. with a single aliquot of endo Glu-C added initially. Lanes 4, 8 and 12, proteolytic digestions performed as described in lanes 3, 7 and 11, respectively but, in addition, a second equal aliquot of the protease added at 2 hrs. Lanes 5, 9 and 13, proteolytic digestions performed at 37°C for 10 hrs. with a single aliquot of endo Glu-C added initially. Lanes 6, 10 and 14, proteolytic digestions performed as described in lanes 5, 9 and 13, respectively but, in addition, a second equal aliquot of the protease added at 2 hrs. Lane 15, the protease control prepared as follows: an aliquot of the protease (equal to that used in the proteolytic digestions performed at an E:S ratio [w/w] of 1:10) was added to 0.1 M NH_5CO_3 pH 7.8 containing 0.1% SDS and incubated at 37°C for 10 hrs., with a second equal aliquot of the protease added at 2 hrs. Protease was inactivated in proteolytic digestions and protease control by heating at 100°C for 20 mins. immediately following incubation at 37°C. SDS-PAGE treated AFP (20 μg) and controls was performed using a 15% (w/v) acrylamide SDS gel and stained with Coomassie Blue. The molecular weight markers indicated in lane A are phosphorylase B (94 Kd), bovine serum albumin (67 Kd), ovalbumin (43 Kd), carbonic anhydrase (30 Kd), soybean trypsin inhibitor (20.1 Kd), and α-lactalbumin (14.4 Kd).

Endoproteinase Glu-C Proteolysis of AFP in Denaturing Buffer

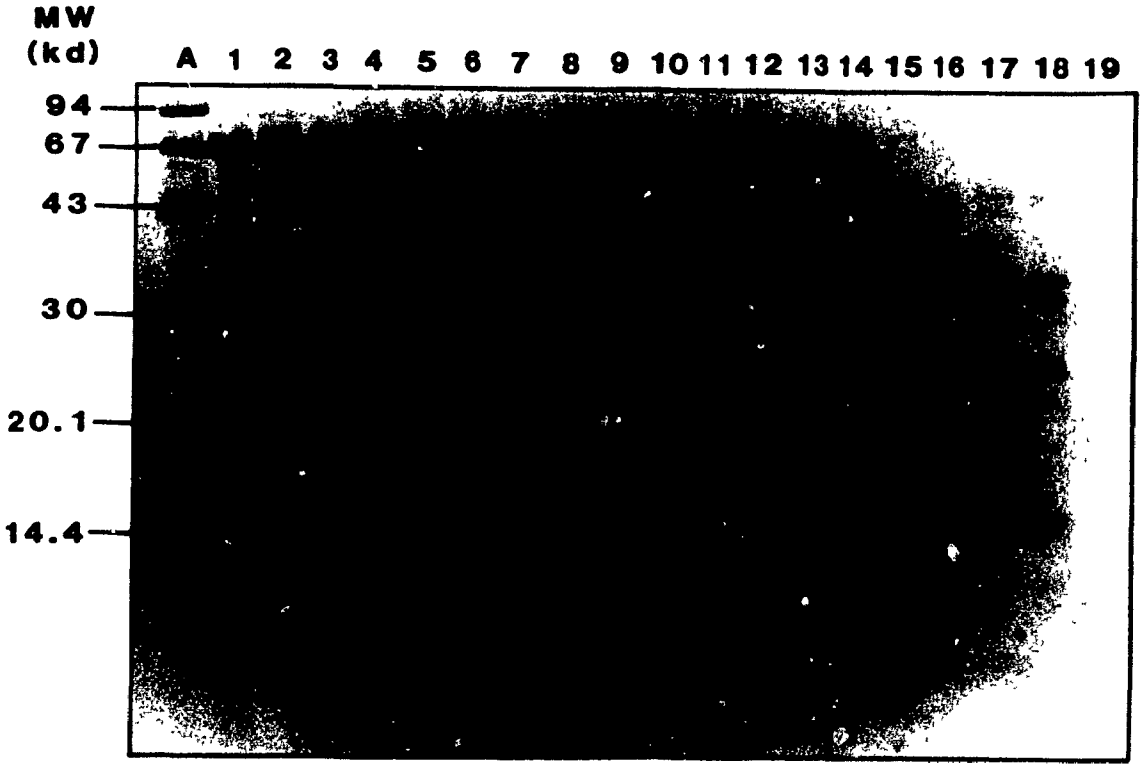


	Conditions	Lane Number
i.	Protease: Endoproteinase Glu-C	
ii.	Buffer: 0.1 M NH_5CO_3, 0.1% SDS, pH 7.8 (denaturing)	1 - 15
iii.	AFP	1
	AFP incubated at 37°C for 10 hrs.	2
iv.	Protease-treated AFP	3 - 14
v.	E:S ratio (w/w) - 1:50	3 - 6
	- 1:25	7 - 10
	- 1:10	11 - 14
vi.	Incubated at 37°C for 4 hrs. with the single aliquot of protease added initially.	3,7,11
vii.	Incubated at 37°C for 10 hrs. with the single aliquot of protease added initially.	4,8,12
viii.	Incubated at 37°C for 4 hrs. with an aliquot of protease added initially and a second equal aliquot of protease added at 2 hrs.	5,9,13
ix.	Incubated at 37°C for 10 hrs. with an aliquot of protease added initially and a second equal aliquot of protease added at 2 hrs.	6,10,14
x.	Protease control incubated at 37°C for 10 hrs.: an aliquot of protease (equal to the aliquot of protease used in the proteolysis of AFP at an E:S ratio 1:10) was added initially to buffer and a second equal aliquot of protease was added at 2 hrs.	15

Figure 4.

Endoproteinase (endo) Lys-C proteolysis of AFP in non-denaturing versus denaturing buffers. SDS-PAGE of endo Lys-C-treated AFP and controls. Lane 1, AFP. Lane 2, AFP incubated at 37°C for 10 hrs. and followed by heating at 100°C for 20 mins. Lanes 3-6 and 11-14, endo Lys-C digestions of AFP carried out in 0.1 M NH_5CO_3 pH 8.5 (non-denaturing buffer) at an E:S ratio (w/w) of 1:100 (lanes 3-6) or 1:25 (lanes 11-14). Lanes 7-10 and 15-18, endo Lys-C digestions of AFP carried out in 0.1 M NH_5CO_3 pH 8.5 containing 0.1% SDS (denaturing buffer) at an E:S (w/w) ratio of 1:100 (lanes 7-10) or 1:25 (lanes 15-18). Lanes 3, 7, 11 and 15, proteolytic digestions performed at 37°C for 4 hrs. with a single aliquot of endo Lys-C added initially. Lanes 5, 9, 13 and 17, proteolytic digestions performed as described in lanes 3, 7, 11 and 15, respectively but, in addition, a second equal aliquot of the protease added at 2 hrs. Lanes 4, 8, 12 and 16, proteolytic digestions performed at 37°C for 10 hrs. with a single aliquot of endo Lys-C added initially. Lanes 6, 10, 14 and 18, proteolytic digestions performed as described in lanes 4, 8, 12 and 16, respectively but, in addition, a second equal aliquot of the protease added at 2 hrs. Lane 19, the protease control performed as described in the legend to Fig. 3, except in 0.1 M NH_5CO_3 pH 8.5 containing 0.1% SDS at an E:S (w/w) ratio of 1:25. Protease inactivation and SDS-PAGE were carried out as outlined in the legend to Fig. 3. Molecular weight markers are indicated in lane A.

Endoproteinase Lys-C Proteolysis of AFP in Non-Denaturing versus Denaturing Buffers

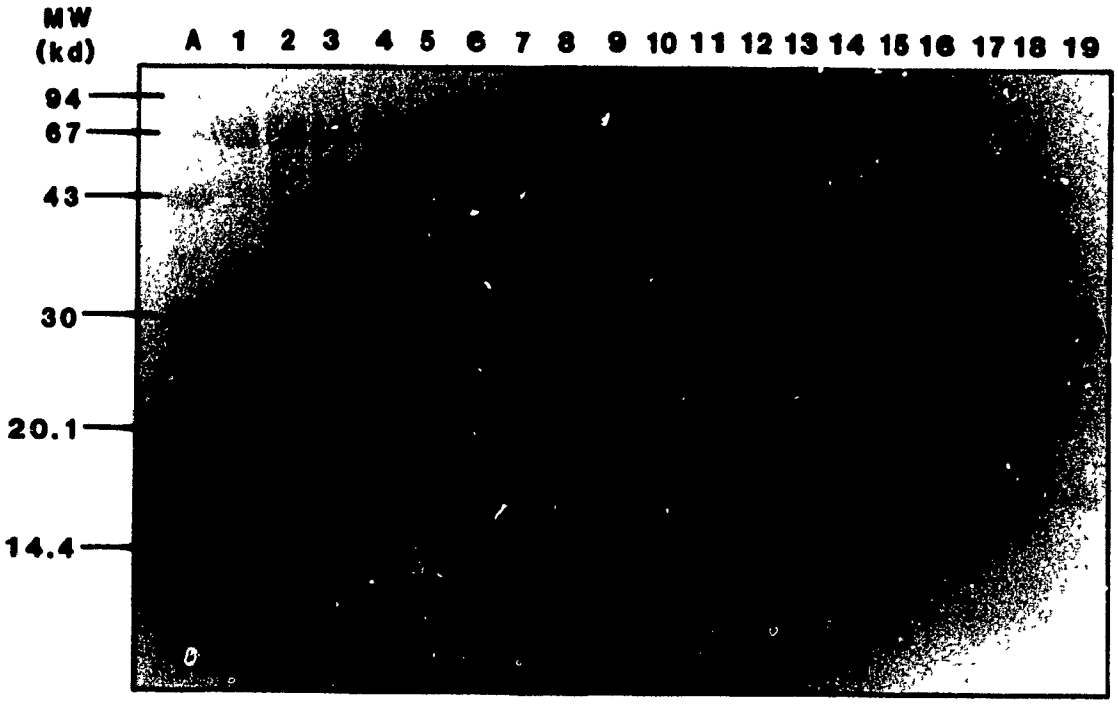


	Conditions	Lane Number
i.	Protease: Endoproteinase Lys-C	
ii.	Buffer: 0.1 M NH_5CO_3, pH 8.5 (non-denaturing)	3-6,11-14
	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5 (denaturing)	1-2,7-10,15-19
iii.	AFP	1
	AFP incubated at 37°C for 10 hrs.	2
iv.	Protease-treated AFP	3 - 18
v.	E:S ratio (w/w) - 1:100	3 - 10
	- 1:25	11 - 18
vi.	Incubated at 37°C for 4 hrs. with the single aliquot of protease added initially.	3,7,11,15
vii.	Incubated at 37°C for 10 hrs. with the single aliquot of protease added initially.	4,8,12,16
viii.	Incubated at 37°C for 4 hrs. with an aliquot of protease added initially and a second equal aliquot of protease added at 2 hrs.	5,9,13,17
ix.	Incubated at 37°C for 10 hrs. with an aliquot of protease added initially and a second equal aliquot of protease added at 2 hrs.	6,10,14,18
x.	Protease control incubated at 37°C for 10 hrs.: an aliquot of protease (equal to the aliquot of protease used in the proteolysis of AFP at an E:S ratio 1:25) was added initially to buffer and a second equal aliquot of protease was added at 2 hrs. ..	19

Figure 5.

Endoproteinase (endo) Arg-C proteolysis of AFP in non-denaturing versus denaturing buffers. SDS-PAGE analysis of endo Arg-C-treated AFP and controls. Lane 1, AFP. Lane 2, AFP incubated at 37°C for 10 hrs. and followed by heating at 100°C for 20 mins. Lanes 3-6 and 11-14, endo Arg-C digestions of AFP carried out in 0.1 M NH_5CO_3 pH 8.5 (non-denaturing buffer) at an E:S (w/w) ratio of 1:25 (lanes 3-6) or 1:10 (lanes 11-14). Lanes 7-10 and 15-18, endo Arg-C digestions of AFP carried out in 0.1 M NH_5CO_3 pH 8.5 containing 0.1% SDS (denaturing buffer) at an E:S (w/w) ratio of 1:25 (lanes 7-10) or 1:10 (lanes 15-18). Lanes 3, 7, 11 and 15, proteolytic digestions performed at 37°C for 4 hrs. with a single aliquot of endo Arg-C added initially. Lanes 5, 9, 13 and 17, proteolytic digestions performed as described in lanes 3, 7, 11, and 15, respectively but, in addition, a second equal aliquot of the protease added at 2 hrs. Lanes 4, 8, 12 and 16, proteolytic digestions performed at 37°C for 10 hrs. with a single aliquot of endo Arg-C added initially. Lanes 6, 10, 14 and 18, proteolytic digestions performed as described in lanes 4, 8, 12 and 16, respectively but, in addition, a second equal aliquot of the protease added at 2 hrs. Lane 19, the protease control, performed as described in the legend to Fig. 3, except in 0.1 M NH_5CO_3 pH 8.5 containing 0.1% SDS. Protease inactivation and SDS-PAGE were carried out as outlined in the legend to Fig. 3. Molecular weight markers are indicated in lane A.

Endoproteinase Arg-C Proteolysis of AFP in Non-Denaturing versus Denaturing Buffers

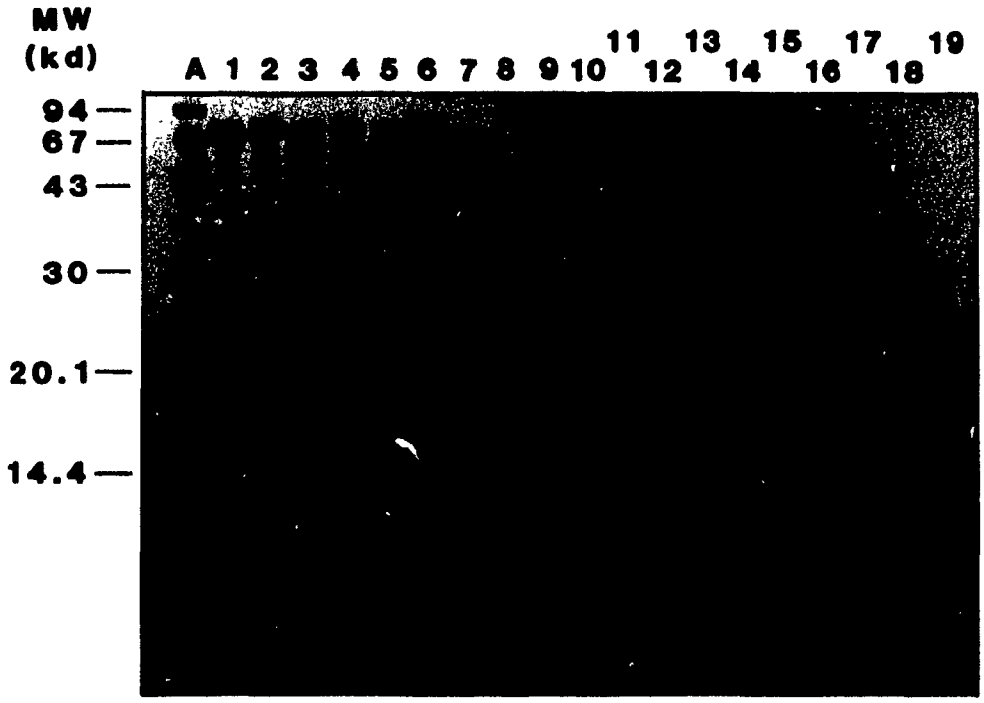


	Conditions	Lane Number
i.	Protease: Endoproteinase Arg-C	
ii.	Buffer: 0.1 M NH_5CO_3, pH 8.5 (non-denaturing)	3-6,11-14
	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5 (denaturing)	1-2,7-10,15-19
iii.	AFP	1
	AFP incubated at 37°C for 10 hrs.	2
iv.	Protease-treated AFP	3 - 18
v.	E:S ratio (w/w) - 1:25	3 - 10
	- 1:10	11 - 18
vi.	Incubated at 37°C for 4 hrs. with the single aliquot of protease added initially.	3,7,11,15
vii.	Incubated at 37°C for 10 hrs. with the single aliquot of protease added initially.	4,8,12,16
viii.	Incubated at 37°C for 4 hrs. with an aliquot of protease added initially and a second equal aliquot of protease added at 2 hrs.	5,9,13,17
ix.	Incubated at 37°C for 10 hrs. with an aliquot of protease added initially and a second equal aliquot of protease added at 2 hrs.	6,10,14,18
x.	Protease control incubated at 37°C for 10 hrs.: an aliquot of protease (equal to the aliquot of protease used in the proteolysis of AFP at an E:S ratio 1:10) was added initially to buffer and a second equal aliquot of protease was added at 2 hrs. ..	19

Figure 6.

Chymotrypsin A_4 proteolysis of AFP in non-denaturing versus denaturing buffers. SDS-PAGE of chymotrypsin A_4-treated AFP and controls. Lane 1, AFP. Lane 2, AFP incubated at 37°C for 10 hrs. and followed by heating at 100°C for 20 mins. Lanes 3-6 and 11-14, chymotrypsin A_4 digestions of AFP carried out in 0.1 M NH_5CO_3 pH 8.5 (non-denaturing condition) at an E:S (w/w) ratio of 1:33 (lanes 3-6) or 1:10 (lanes 11-14). Lanes 3, 7, 11 and 15, proteolytic digestions performed at 37°C for 4 hrs. with a single aliquot of chymotrypsin A_4 added initially. Lanes 5, 9, 13 and 17, proteolytic digestions performed as described in lanes 3, 7, 11 and 15, respectively but, in addition, a second equal aliquot of the protease added at 2 hrs. Lanes 4, 8, 12 and 16, proteolytic digestions were performed at 37°C for 10 hrs. with a single aliquot of chymotrypsin A_4 added initially. Lanes 6, 10, 14 and 18, proteolytic digestions performed as described in lanes 4, 8, 12 and 16, respectively but, in addition, a second equal aliquot of the protease added at 2 hrs. Lane 19, the protease control, performed as described in the legend to Fig. 3, except in 0.1 M NH_5CO_3 pH 8.5 containing 0.1% SDS. Protease inactivation and SDS-PAGE were carried out as outlined in the legend to Fig. 3. Molecular weight markers are indicated in lane A.

Chymotrypsin A_4 Proteolysis of AFP in Non-Denaturing versus Denaturing Buffers

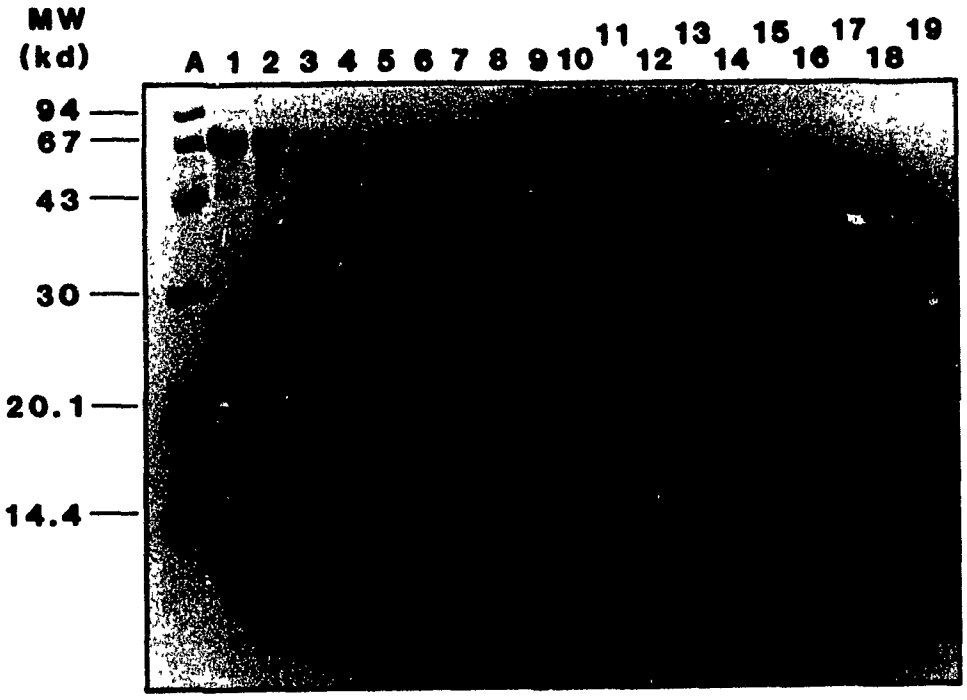


	Conditions	Lane Number
i.	Protease: Chymotrypsin A_4	
ii.	Buffer: 0.1 M NH_5CO_3, pH 8.5 (non-denaturing)	3-6,11-14
	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5 (denaturing)	1-2,7-10,15-19
iii.	AFP	1
	AFP incubated at 37°C for 10 hrs.	2
iv.	Protease-treated AFP	3 - 18
v.	E:S ratio (w/w) - 1:33	3 - 10
	- 1:10	11 - 18
vi.	Incubated at 37°C for 4 hrs. with the single aliquot of protease added initially	3,7,11,15
vii.	Incubated at 37°C for 10 hrs. with the single aliquot of protease added initially	4,8,12,16
viii.	Incubated at 37°C for 4 hrs. with an aliquot of protease added initially and a second equal aliquot of protease added at 2 hrs	5,9,13,17
ix.	Incubated at 37°C for 10 hrs. with an aliquot of protease added initially and a second equal aliquot of protease added at 2 hrs	6,10,14,18
x.	Protease control incubated at 37°C for 10 hrs.: an aliquot of protease (equal to the aliquot of protease used in the proteolysis of AFP at an E:S ratio 1:10) was added initially to buffer and a second equal aliquot of protease was added at 2 hrs ...	19

Figure 7.

Elastase proteolysis of AFP carried out during a 4 or 10 hour incubation period in non-denaturing versus denaturing buffers. SDS-PAGE of elastase-treated AFP and controls. Lane 1, AFP. Lane 2, AFP incubated at 37°C for 10 hrs. followed by heating at 100°C for 20 mins. Lanes 3-6 and 11-14, elastase digestions of AFP carried out in 0.1 M NH_5CO_3 pH 8.5 (non-denaturing condition) at an E:S (w/w) ratio of 1:25 (lanes 3-6) or 1:10 (lanes 11-14). Lanes 3, 7, 11 and 15, proteolytic digestions performed at 37°C for 4 hrs. with a single aliquot of elastase added initially. Lanes 5, 9, 13 and 17, proteolytic digestions performed as described in lanes 3, 7, 11 and 15, respectively but, in addition, a second equal aliquot of the protease added at 2 hrs. Lanes 4, 8, 12 and 16, proteolytic digestions performed at 37°C for 10 hrs. with a single aliquot of elastase added initially. Lanes 6, 10, 14 and 18, proteolytic digestions performed as described in lanes 4, 8, 12 and 16, respectively but, in addition, a second equal aliquot of the protease added at 2 hrs. Lane 19, the protease control, performed as described in the legend to Fig. 3, except in 0.1 M NH_5CO_3 pH 8.5 containing 0.1% SDS. Protease inactivation and SDS-PAGE were carried out as outlined in the legend to Fig. 3. Molecular weight markers are indicated in lane A.

Elastase Proteolysis of AFP Carried Out During a 4 or 10 Hour Incubation Period in Non-Denaturing versus Denaturing Buffers

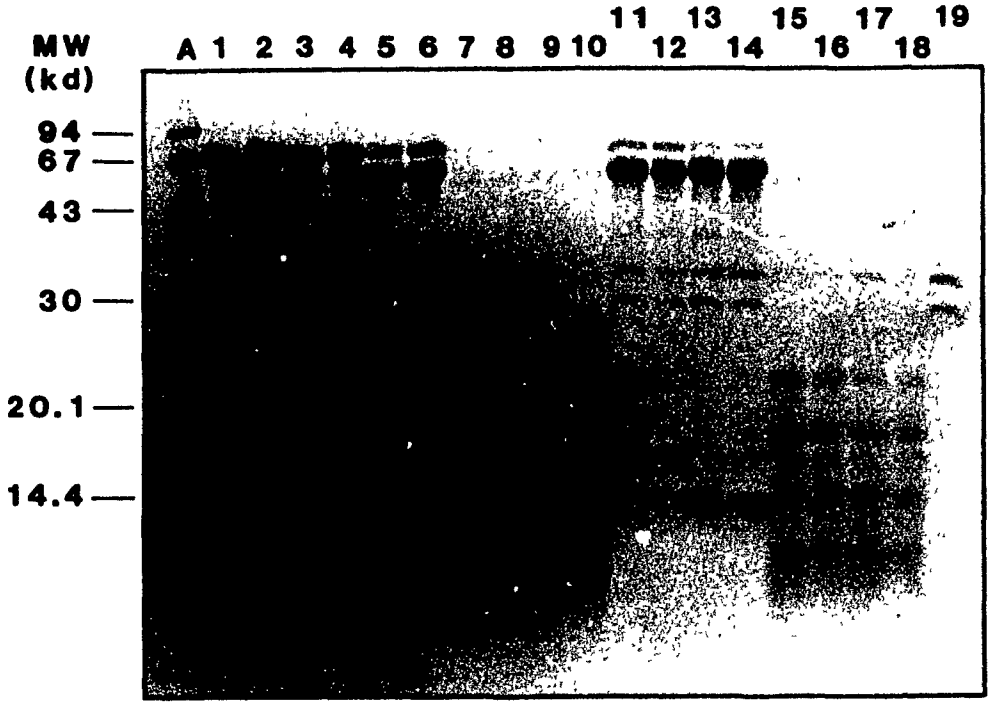


	Conditions	Lane Number
i.	Protease: Elastase	
ii.	Buffer: 0.1 M NH_5CO_3, pH 8.5 (non-denaturing)	3-6,11-14
	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5 (denaturing)	1-2,7-10,15-19
iii.	AFP	1
	AFP incubated at 37°C for 10 hrs.	2
iv.	Protease-treated AFP	3 - 18
v.	E:S ratio (w/w) - 1:25	3 - 10
	- 1:10	11 - 18
vi.	Incubated at 37°C for 4 hrs. with the single aliquot of protease added initially	3,7,11,15
vii.	Incubated at 37°C for 10 hrs. with the single aliquot of protease added initially	4,8,12,16
viii.	Incubated at 37°C for 4 hrs. with an aliquot of protease added initially and a second equal aliquot of protease added at 2 hrs.	5,9,13,17
ix.	Incubated at 37°C for 10 hrs. with an aliquot of protease added initially and a second equal aliquot of protease added at 2 hrs.	6,10,14,18
x.	Protease control incubated at 37°C for 10 hrs.: an aliquot of protease (equal to the aliquot of protease used in the proteolysis of AFP at an E:S ratio 1:10) was added initially to buffer and a second equal aliquot of protease was added at 2 hrs. ..	19

Figure 8.

Elastase proteolysis of AFP carried out during a 1 or 2 hour incubation period in non-denaturing versus denaturing buffers. SDS-PAGE of elastase-treated AFP and controls. Lane 1, AFP. Lane 2, AFP incubated at 37°C for 2 hrs. and followed by heating at 100°C for 20 mins. Lanes 3-6 and 11-14, elastase digestions of AFP carried out in 0.1 M NH_5CO_3 pH 8.5 (non-denaturing buffer) at an E:S (w/w) ratio of 1:25 (lanes 3-6) or 1:10 (lanes 11-14). Lanes 7-10 and 15-18, elastase digestions of AFP carried out in 0.1 M NH_5CO_3 pH 8.5 containing 0.1% SDS (denaturing buffer) at an E:S (w/w) ratio of 1:25 (lanes 7-10) or 1:10 (lanes 15-18). Lanes 3, 7, 11 and 15, proteolytic digestions performed at 37°C for 1 hr. with a single aliquot of elastase added initially. Lanes 5, 9, 13 and 17, proteolytic digestions performed as described in lanes 3, 7, 11 and 15, respectively but, in addition, a second equal aliquot of the protease added at 30 mins. Lanes 4, 8, 12 and 16, proteolytic digestions performed at 37°C for 2 hrs. with a single aliquot of elastase added initially. Lanes 6, 10, 14 and 18, proteolytic digestions performed as described in lanes 4, 8, 12 and 16, respectively but, in addition, a second equal aliquot of the protease added at 30 mins. Lane 19, the protease control which was prepared as follows: an aliquot of the protease (equal to that used in the proteolytic digestions performed at an E:S [w/w] ratio of 1:10) was added to 0.1 M NH_5CO_3 pH 8.5 containing 0.1% SDS and incubated at 37°C for 2 hrs. with a second equal aliquot of the protease added at 30 mins. Protease inactivation and SDS-PAGE were carried out as outlined in the legend to Fig. 3. Molecular weight markers are indicated in lane A.

Elastase Proteolysis of AFP Carried Out During a 1 or 2 Hour Incubation Period in Non-Denaturing versus Denaturing Buffers



	Conditions	Lane Number
i.	Protease: Elastase	
ii.	Buffer: 0.1 M NH_5CO_3, pH 8.5 (non-denaturing)	3-6,11-14
	0.1 M NH_5CO_3, 0.1% SDS, pH 8.5 (denaturing)	1-2,7-10,15-19
iii.	AFP	1
	AFP incubated at 37°C for 2 hrs.	2
iv.	Protease-treated AFP	3 - 18
v.	E:S ratio (w/w) - 1:25	3 - 10
	- 1:10	11 - 18
vi.	Incubated at 37°C for 1 hr. with the single aliquot of protease added initially	3,7,11,14
vii.	Incubated at 37°C for 2 hrs. with the single aliquot of protease added initially	4,8,12,16
viii.	Incubated at 37°C for 1 hr. with an aliquot of protease added initially and a second equal aliquot of protease added at 30 mins.	5,9,13,17
ix.	Incubated at 37°C for 2 hrs. with an aliquot of protease added initially and a second equal aliquot of protease added at 30 mins.	6,10,14,18
x.	Protease control incubated at 37°C for 2 hrs.: an aliquot of protease (equal to the aliquot of protease used in the proteolysis of AFP at an E:S ratio 1:10) was added initially to buffer and a second equal aliquot of protease was added at 2 hrs. ..	19

Figure 9.

SDS-PAGE analysis of endo Glu-C, papain, trypsin, chymotrypsin A_4 and thermolysin digestions of AFP carried out under the optimum conditions determined and summarized in Table 3. A set of three lanes is bracketed for each protease, endo Glu-C, papain, trypsin, chymotrypsin A_4 and thermolysin, as indicated. Each set of three lanes (from left to right) consists of: AFP treated with the indicated protease in non-denaturing (ND) and denaturing (D) buffers under optimum conditions determined (shown in Table 3), and the corresponding protease (P, in excess of its concentration in the digestions), respectively. Included are the molecular weight markers (for description see the legend to Fig. 3) and mouse (Mo)AFP that has been processed in an identical manner as protease-treated AFP, but without protease. SDS-PAGE was performed using a 15% (w/v) acrylamide resolving gel. Gel was stained with Coomassie Blue.

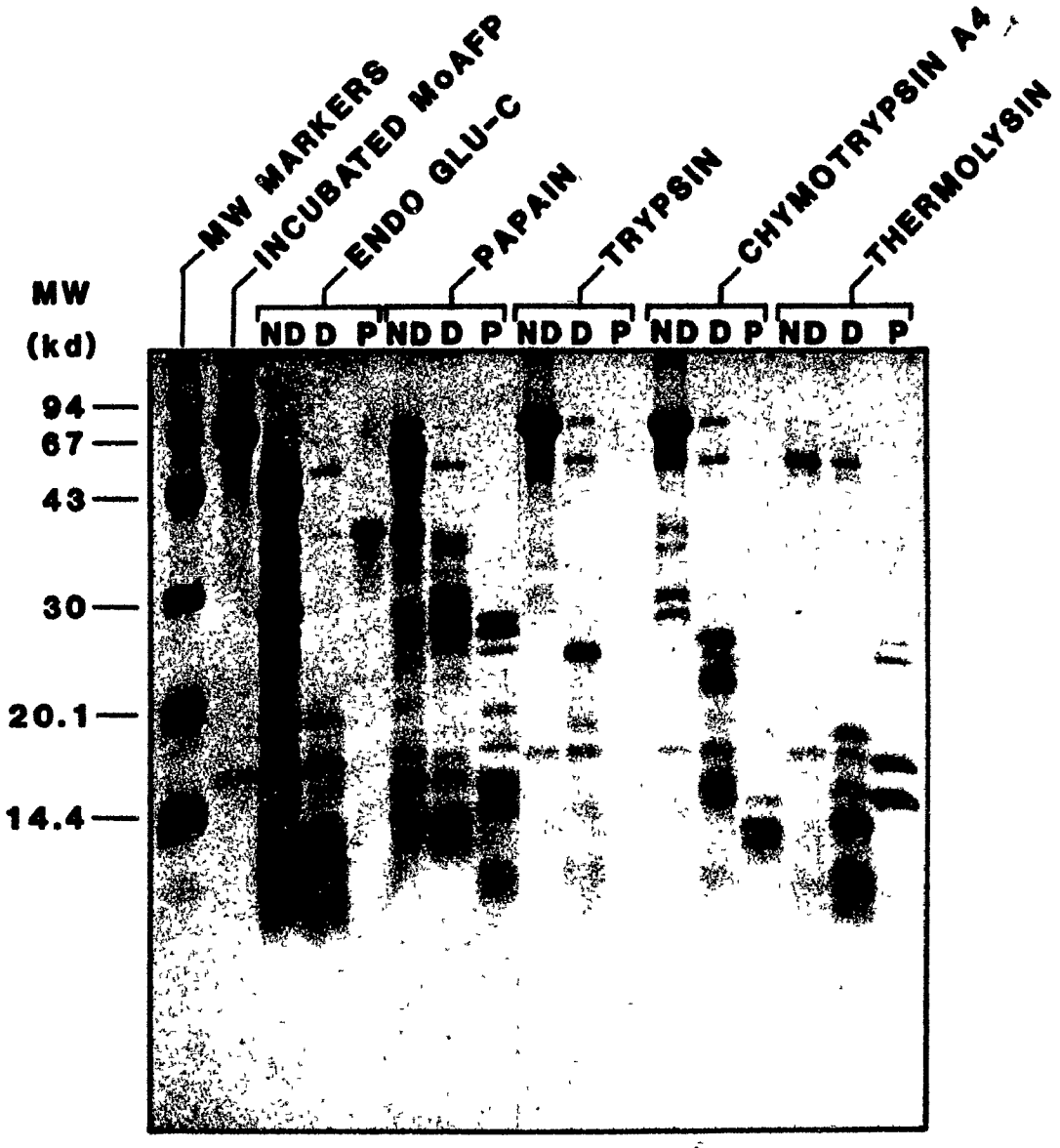

MW MARKERS
INCUBATED MoAFP
ENDO GLU-C
PAPAIN
TRYPSIN
CHYMOTRYPSIN A4
THERMOLYSIN
MW
(kd)
ND D P ND D P ND D P ND D P ND D P
94
67
43
30
20.1
14.4

Figure 10.

SDS-PAGE analysis of elastase digestion of AFP performed under optimum conditions determined and summarized in Table 3. A set of three lanes is bracketed and these lanes are (from left to right): elastase-treated AFP in non-denaturing (ND) and denaturing (D) buffers under optimum conditions determined (described in Table 3), and elastase (P, in excess of its concentration in the digestions), respectively. Included are the molecular weight markers (for description see the legend to Fig. 3) and mouse (Mo)AFP that has been processed in an identical manner as the protease-treated AFP, but without protease. SDS-PAGE was performed using a 15% (w/v) acrylamide resolving gel. Gel was stained with Coomassie Blue.

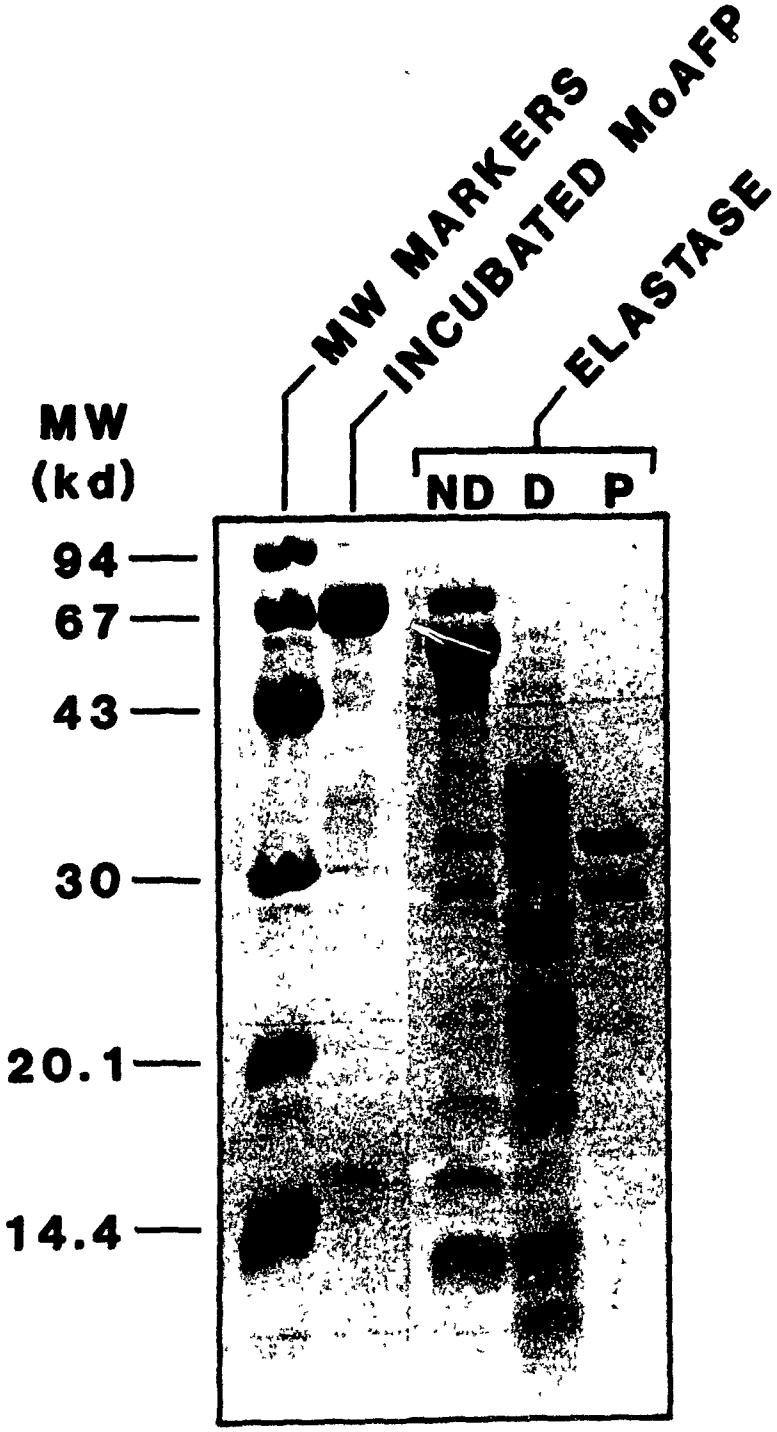

MW MARKERS
INCUBATED MoAFP
ELASTASE
MW
(kd)
ND D P
94
67
43
30
20.1
14.4

Figure 11.

SDS-PAGE analysis of endo Arg-C and endo Lys-C digestions of AFP carried out under optimum conditions determined and summarized in Table 3. A set of three lanes is bracketed for the proteases endo Arg-C and endo Lys-C, as indicated. Each consists of three lanes (from left to right): AFP treated with the indicated protease in non-denaturing (ND) and denaturing (D) buffer under optimum parameters determined (see Table 3), and the corresponding protease control (P, in excess of its concentration in the digests), respectively. Included are the molecular weight markers (for description see the legend to Fig. 3) and AFP that has been processed in an identical manner as the protease-treated AFP, but without protease. SDS-PAGE was performed using a 15% (w/v) acrylamide resolving gel. Gel was stained with Coomassie Blue.

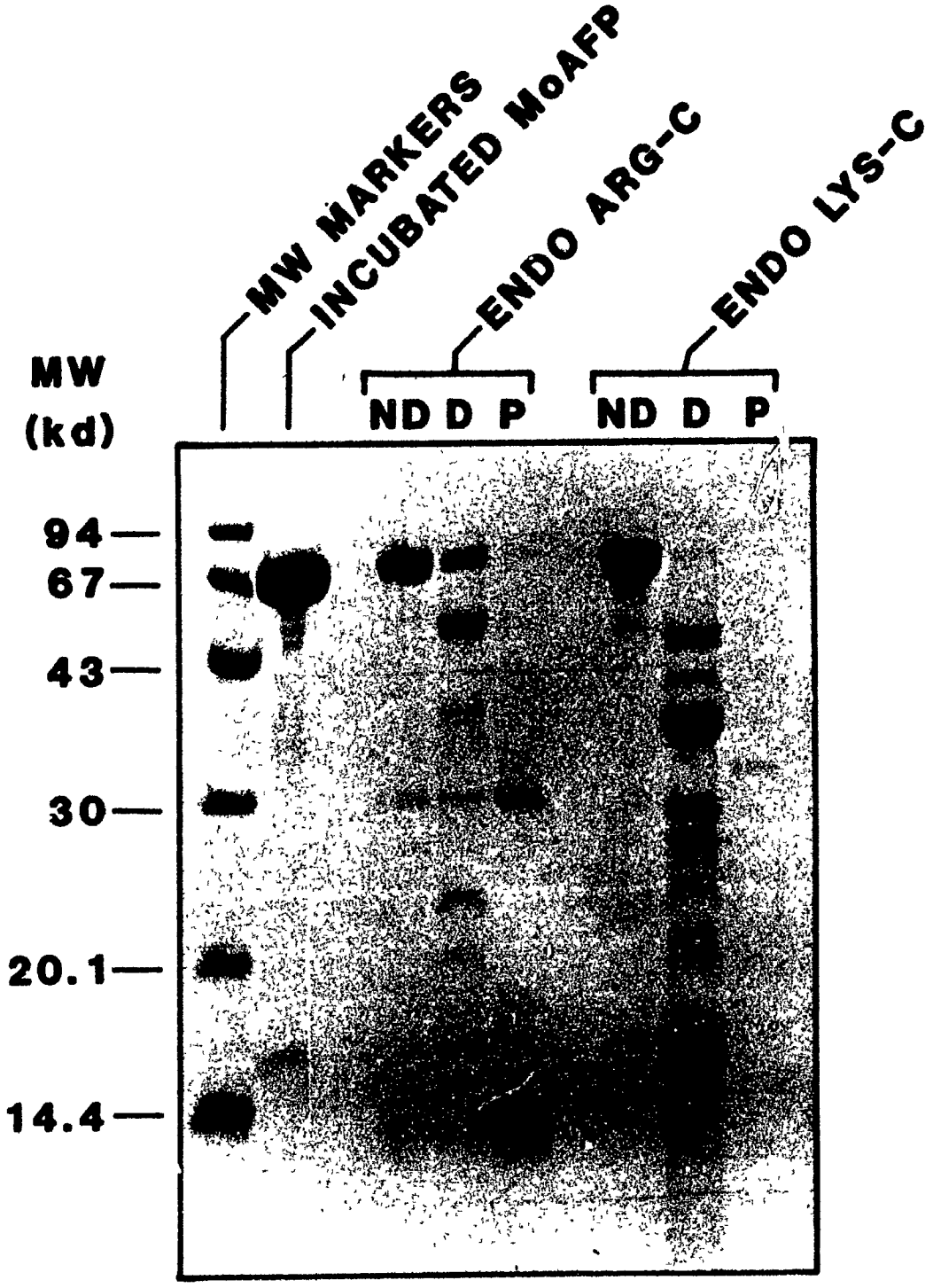

MW MARKERS
INCUBATED MoAFP
ENDO ARG-C
ENDO LYS-C
MW
(kd)
ND D P
ND D P
94
67
43
30
20.1
14.4

Figure 12.

SDS-PAGE analysis of bromelain and pronase digestions of AFP performed under optimum conditions determined and summarized in Table 3. A set of three lanes is bracketed for each protease, bromelain and pronase, as indicated. Each set of three lanes (from left to right) consists of: AFP treated with the indicated protease in non-denaturing (ND) and denaturing (D) buffer under optimum conditions determined (shown in Table 3), and the corresponding protease (P, in excess of its concentration in the digests), respectively. Included are the molecular weight markers (for description see the legend to Fig. 3) and mouse (Mo)AFP that has been processed in an identical manner as the protease-treated AFP solutions, but without the protease. SDS-PAGE was performed using a 15% (w/v) acrylamide resolving gel. Gel was stained with Coomassie Blue.

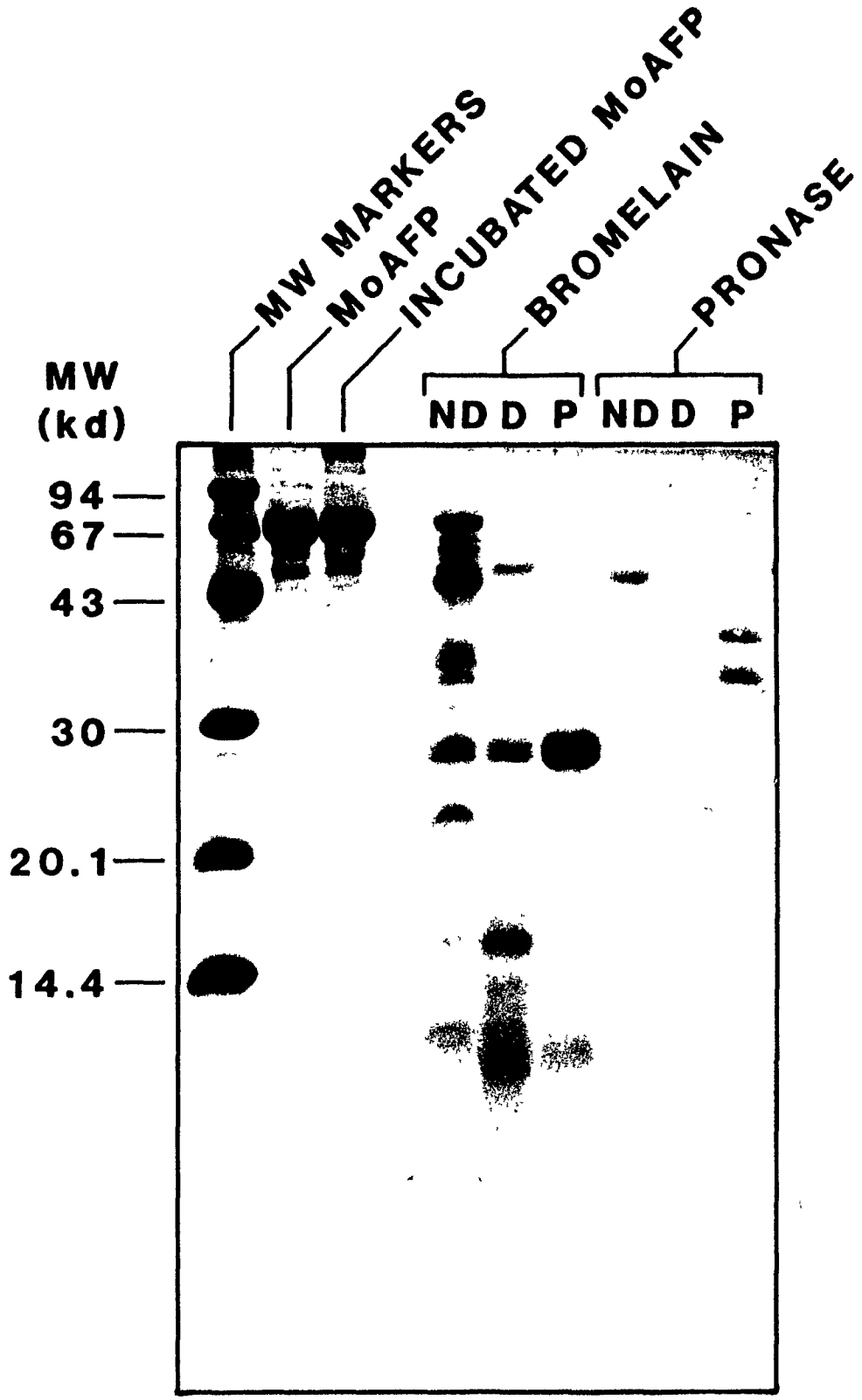
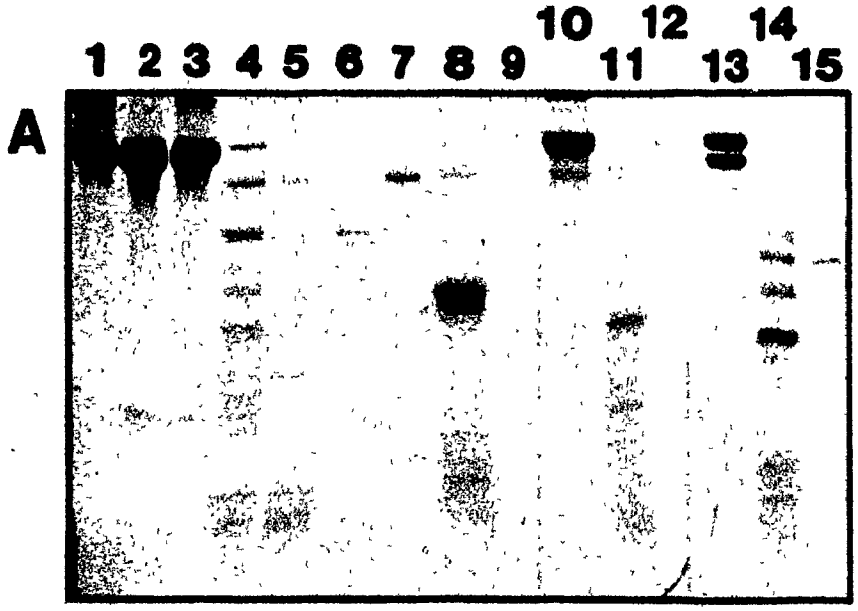

MW MARKERS
MoAFP
INCUBATED MoAFP
BROMELAIN
PRONASE
MW
(kd)
ND D P ND D P
94
67
43
30
20.1
14.4

Figure 13.

SDS-PAGE analysis and corresponding immunoblot of endo-Glu-C, papain, trypsin and elastase digestions of AFP carried out under optimum conditions determined and summarized in Table 3. A, SDS-PAGE analysis of AFP treated separately with endo Glu-C, papain, trypsin and elastase in non-denaturing (ND) and denaturing (D) buffers under the optimum conditions determined and summarized in Table 3. Lane 1, MoALB. Lane 2, AFP. Lane 3, AFP processed in an identical manner as protease-treated AFP, but without protease. Lane 4-6, endo Glu-C-treated AFP in ND and D buffer, and endo Glu-C control, respectively. Lane 7-9, papain-treated AFP in ND and D buffer, and papain control, respectively. Lane 10-12, trypsin-treated AFP in ND and D buffers, and trypsin control, respectively. Lane 13-15, elastase-treated AFP in ND and D buffers, and elastase control, respectively. SDS-PAGE was performed using a 15% (w/v) acrylamide resolving mini-slab gel. Gel was stained with Coomassie Blue. **B,** Immunoblot corresponding to the SDS-polyacrylamide gel displayed in A. The immunoblot was performed using monospecific anti-MoAFP as described in Materials and Methods. Lanes 1-15 of B are as described in the legend to A. However, the samples in B were set at a concentration representing a 1/100 dilution of those samples described in the legend to A.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
A

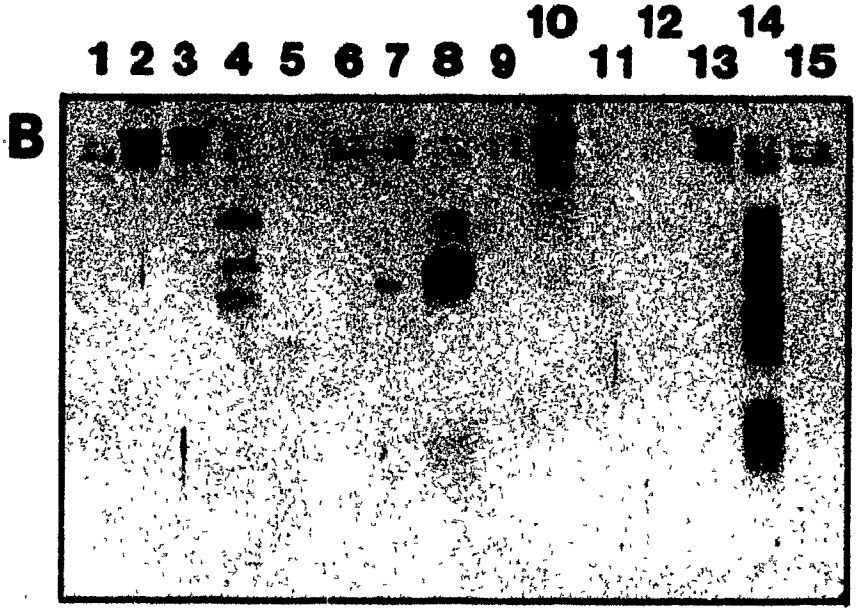
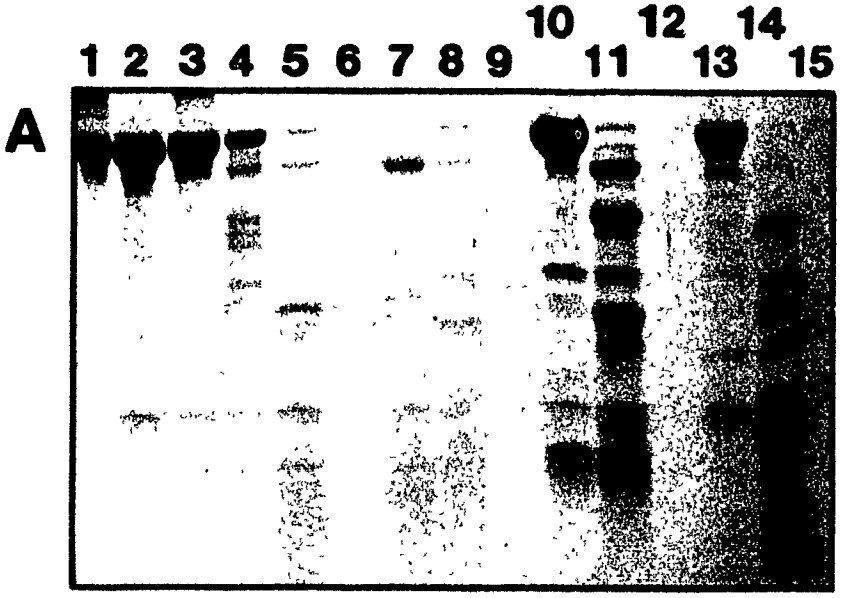

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B

Figure 14.

SDS-PAGE analysis and corresponding immunoblot of chymotrypsin A_4, thermolysin, endo Arg-C and endo Lys-C digestions of AFP carried out under optimum conditions determined and summarized in Table 3. A, SDS-PAGE analysis of AFP treated separately with chymostrypsin A_4, thermolysin, endo Arg-C and endo Lys-C in non-denaturing (ND) and denaturing (D) buffers under the optimum conditions determined and described in Table 3. Lane 1, MoALB. Lane 2, AFP. Lane 3, AFP processed in an identical manner as protease-treated AFP, but without protease. Lane 4-6, chymotrypsin A_4-treated AFP in ND and D buffers, and chymostrypsin A_4 control, respectively. Lane 7-9, thermolysin-treated AFP in ND and D buffers, and thermolysin control, respectively. Lane 10-12, endo Arg-C-treated AFP in ND and D buffers, and endo Arg-C control, respectively. Lane 13-15, endo Lys-C-treated AFP in ND and D buffers, and endo Lys-C control, respectively. SDS-PAGE was performed using a 15% (w/v) acrylamide resolving mini-slab gel. Protein bands were stained by Coomassie Blue. **B,** Immunoblot corresponding to the SDS-polyacrylamide gel displayed in A. The immunoblot was performed using monospecific anti-MoAFP as described in Materials and Methods. Lanes 1-15 of B are as described in the legend to A. However, the samples in B were set at a concentration representing a 1/100 dilution of those samples described in the legend to A.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

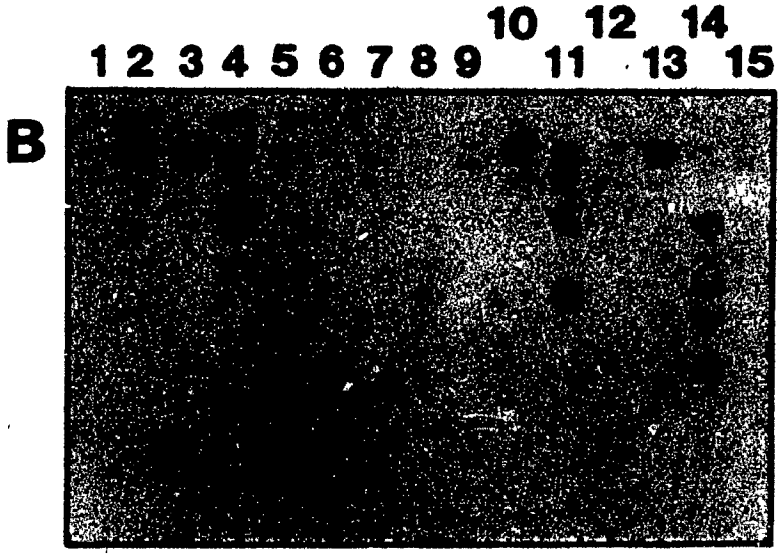

B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 15.

SDS-PAGE analysis and corresponding immunoblot of bromelain and pronase digestions of AFP carried out under optimum conditions determined and summarized in Table 3. A, SDS-PAGE analysis of AFP treated separately with bromelain and pronase in non-denaturing (ND) and denaturing (D) buffers under the optimum conditions determined and summarized in Table 3. Lane 1, MoALB. Lane 2, AFP. Lane 3, AFP processed in an identical manner as protease-treated AFP, but without protease. Lane 4-6, bromelain-treated AFP in ND and D buffers, and bromelain control, respectively. Lane 7-9, pronase-treated AFP in ND and D buffers, and pronase control, respectively. SDS-PAGE was performed using a 15% (w/v) acrylamide resolving mini-slab gel. Protein bands were stained by Coomassie Blue. **B,** Immunoblot corresponding to the SDS-polyacrylamide gel displayed in A. The immunoblot was performed using monospecific anti-MoAFP as described in Materials and Methods. Lanes 1-9 of B are as described in the legend to A. The samples in B were set at a concentration representing a 1/100 dilution of those samples described in the legend to A.

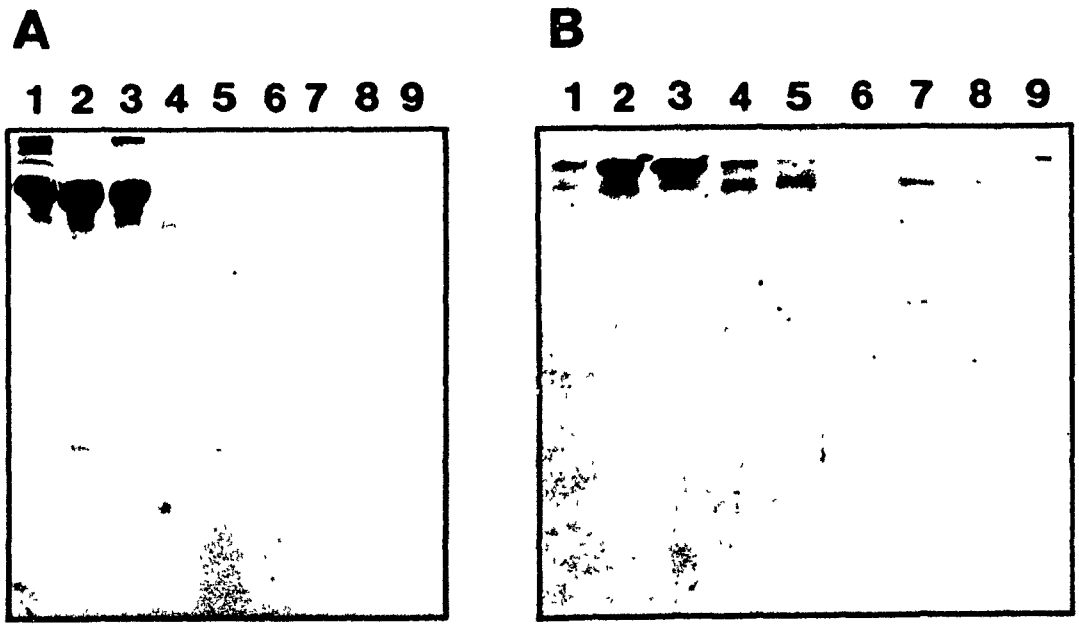
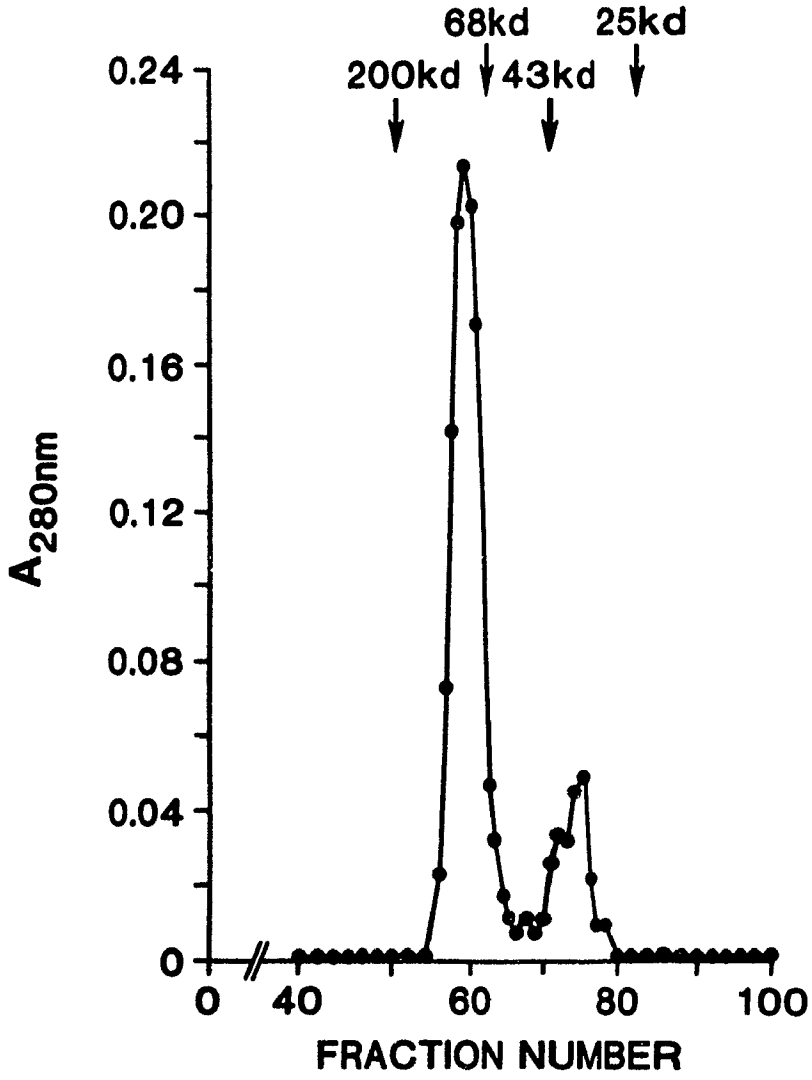

A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9

Figure 16.

Gel filtration of elastase-treated AFP on Sephacryl S-200 column. A sample (3.3 ml) containing 30 mg of elastase-treated AFP was applied and eluted as described in Materials and Methods. The elution profile is displayed in **A.** Several calibration standards are also indicated on the profile in **A.** An aliquot (15 µl) of each fraction which eluted from the column in the peak areas, as well as fractions eluting before and after the peaks were also analyzed by SDS-PAGE performed using a 15% (w/v) acrylamide resolving mini-slab gel. **B** shows SDS-PAGE analysis of certain fractions eluting at the peak areas displayed in **A** which are representative of the type of partial separation obtained: lane 1, molecular weight markers (top to bottom) are phosphorylase B (94 Kd), bovine serum albumin (67 Kd), ovalbumin (43 Kd), carbonic anhydrase (30 Kd), soybean trypsin inhibitor (20.1 Kd) and α-lactalbumin (14.4 Kd), respectively; lane 2, AFP; lane 3, elastase-treated AFP; lane 4, fraction 57 (representative of the SDS gel pattern obtained with fractions 58-71); lane 5, fraction 72 (representative of the SDS gel pattern obtained with fractions 73-76); lane 6, fraction 77 (representative of the SDS gel pattern obtained with fractions 78-79); and lane 7, elastase.

A


68kd
25kd
200kd
43kd
0.24
0.20
0.16
0.12
0.08
0.04
0
A_{280nm}
0
40
60
80
100
FRACTION NUMBER

B

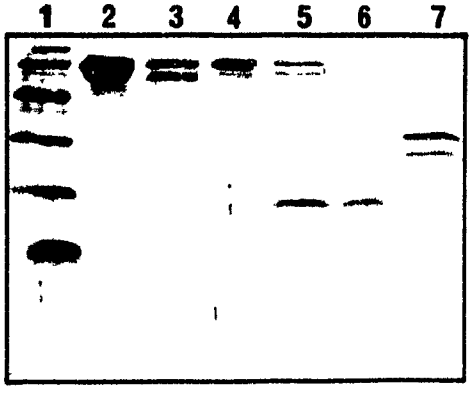
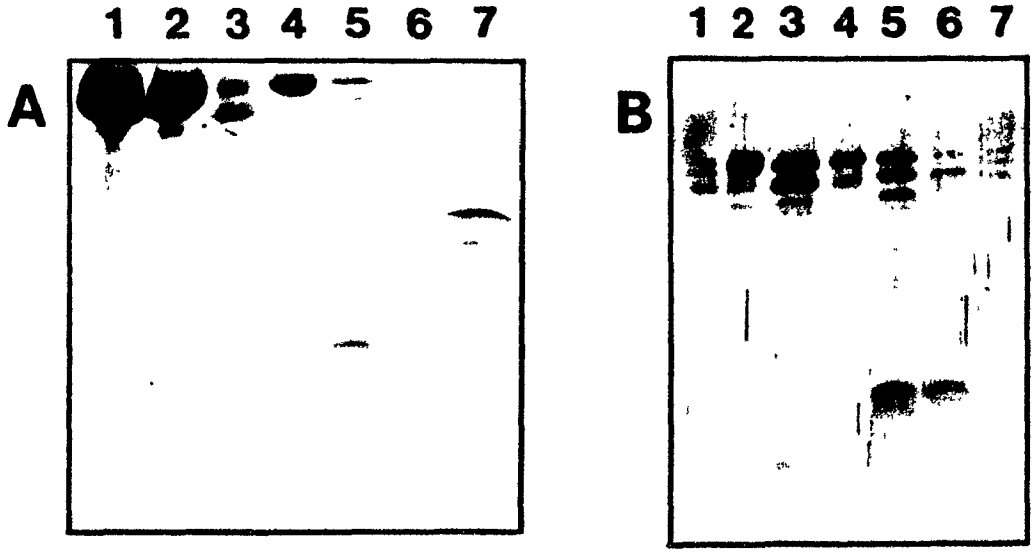
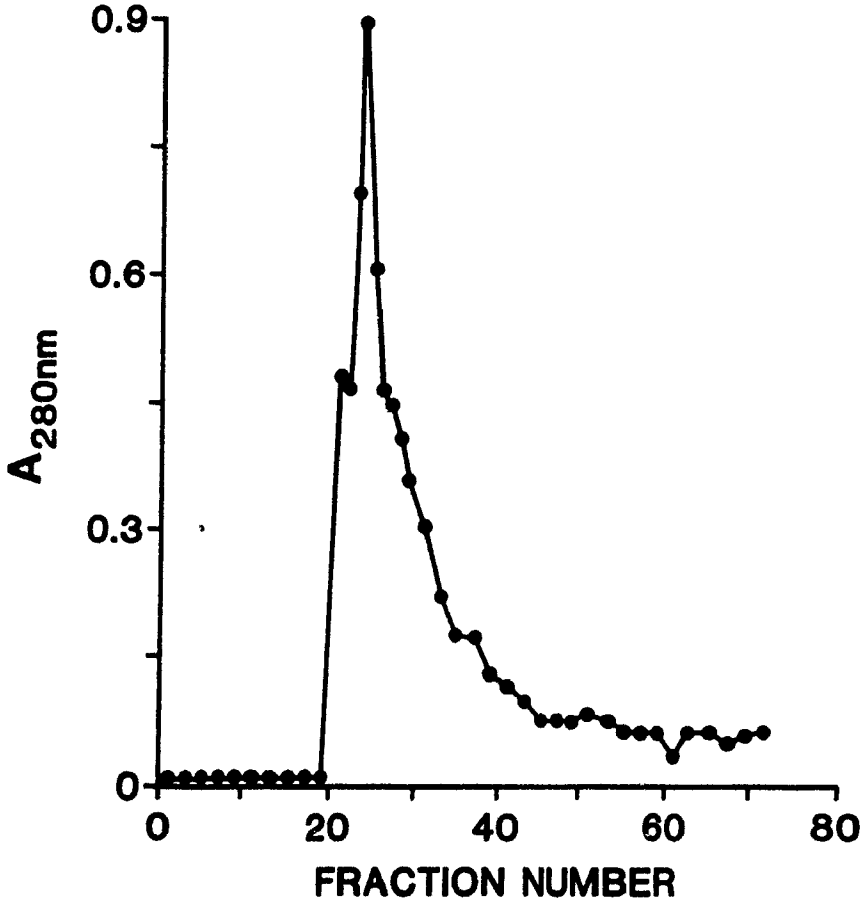

1
2
3
4
5
6
7

Figure 17.

SDS-PAGE analysis and corresponding immunoblot of fractions obtained from gel filtration chromatography of elastase-treated AFP on Sephacryl S-200 column. **A,** SDS-PAGE analysis of fractions (15 µl aliquot of each) representative of the kind of separation obtained by gel filtration chromatography of elastase-treated AFP. Lane 1, MoALB. Lane 2, AFP. Lane 3, elastase-treated AFP. Lane 4, fraction 57. Lane 5, fraction 72. Lane 6, fraction 77. Lane 7, elastase. SDS-PAGE was performed using a 15% (w/v) acrylamide resolving mini-slab gel. Gel was stained with Coomassie Blue. **B,** Immunoblot corresponding to the SDS-polyacrylamide gel displayed in **A.** The immunoblot was performed using monospecific anti-MoAFP as described in Materials and Methods. Lanes 1-7 of **B** are as described in the legend to **A.** However, the samples were set at a concentration representing a 1/100 dilution of those samples described above in the legend to **A.**


1 2 3 4 5 6 7
A
1 2 3 4 5 6 7
B

A


0.9
0.6
0.3
0
A_{280nm}
0
20
40
60
80
FRACTION NUMBER

B

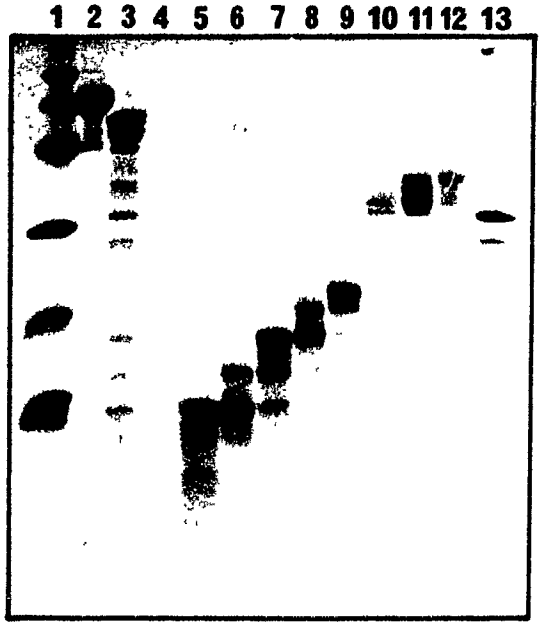
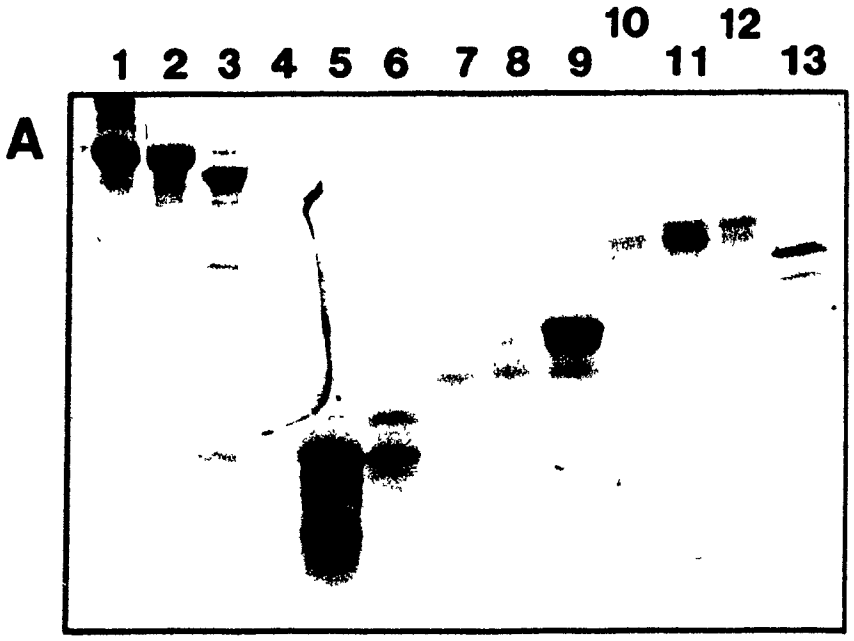

1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 19.

SDS-PAGE analysis and the corresponding immunoblot of samples containing partially isolated cleavage products obtained from successive zone elution of elastase-treated AFP by preparative SDS-polyacrylamide gel disc electrophoresis. A, SDS-PAGE analysis carried out on an aliquot (5 µl) of each of the samples 1-9 (see Table 5) obtained from the succesive zone elution of elastase-treated AFP. Lane 1, MoALB. Lane 2, AFP. Lane 3, elastase-treated AFP. Lane 4, sample 1. Lane 5, sample 2. Lane 6, sample 3. Lane 7, sample 4. Lane 8, sample 5. Lane 9, sample 6. Lane 10, sample 7. Lane 11, sample 8. Lane 12, sample 9. Lane 13, elastase. SDS-PAGE was performed using a 15% (w/v) acrylamide resolving mini-slab gel. Gel was stained with Coomassie Blue. **B,** Immunoblot corresponding to the SDS-polyacrylamide gel displayed in **A.** The immunoblot was performed using monospecific anti-MoAFP as described in Materials and Methods. Lanes 1-13 of **B** are as described in the legend to **A.** However, the samples in B were set at a concentration representing a 1/100 dilution of the samples described in the legend to **A.**


1 2 3 4 5 6 7 8 9 10 11 12 13
A

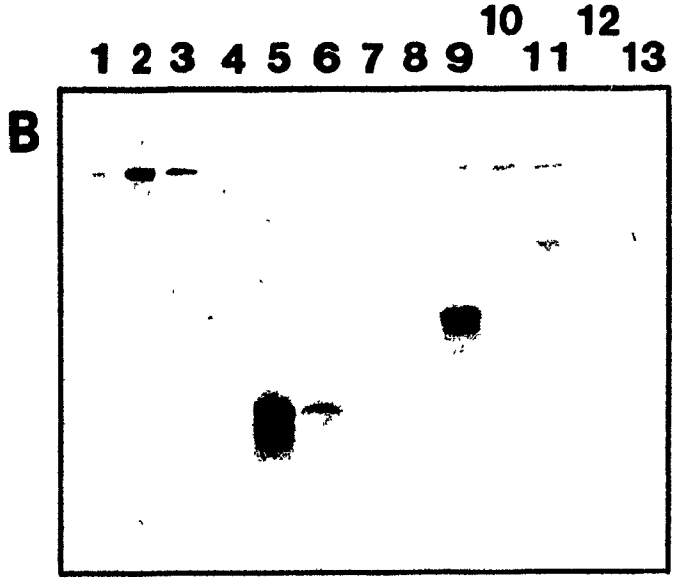

1 2 3 4 5 6 7 8 9 10 11 12 13
B

Figure 20.

Analytical chromatography of endo Glu-C-treated AFP by reverse-phase HPLC. Large-scale limited proteolytic digestion of AFP by endo Glu-C was carried out as described in Materials and Methods. A 15 µl (300 µg) aliquot was injected onto a C_{18} µBondapak reverse-phase column. The column was equilibrated with 5% acetonitrile in water containing 0.1% TFA and eluted in 65 mins. with an acetonitrile gradient containing 0.1% TFA as depicted in **A**, at a flow rate of 1.0 ml/min. Proteins eluting from the column were monitored spectrophotometrically by absorbance at 206 nm and 280 nm.

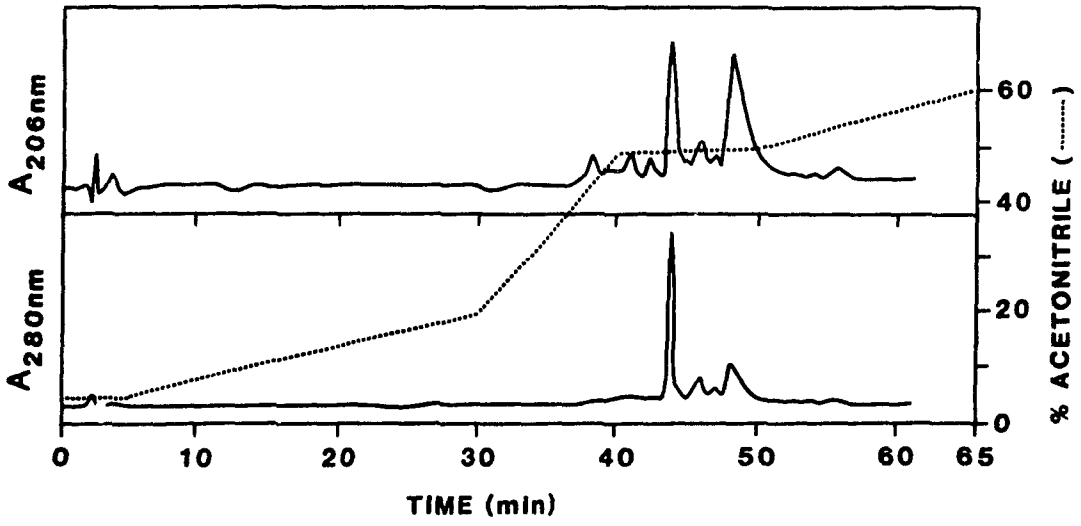
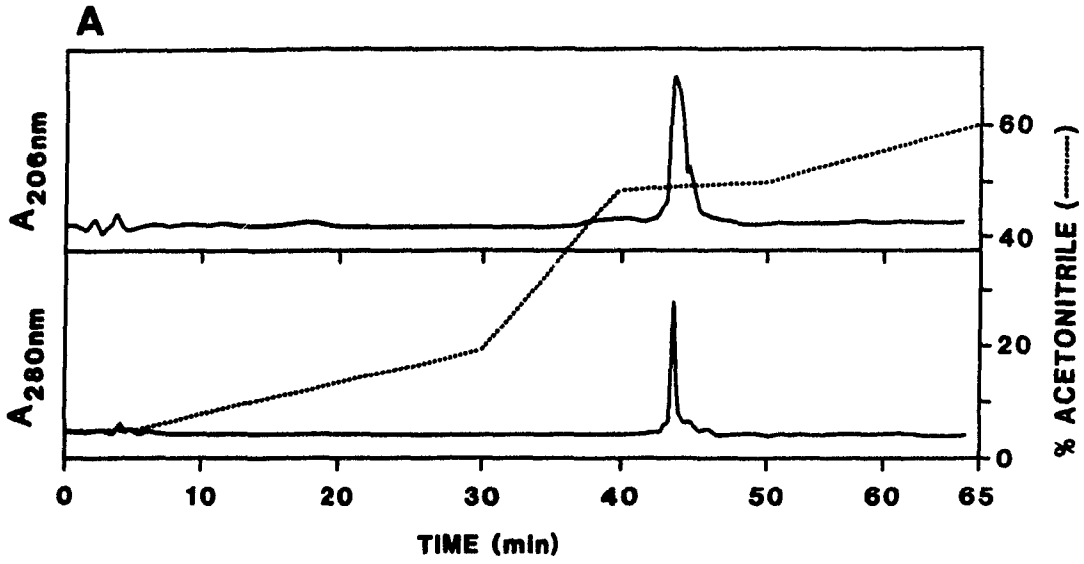

A206nm
A280nm
60
40
20
0
% ACETONITRILE (------)
0
10
20
30
40
50
60
65
TIME (min)

Figure 21.

Analytical chromatography of AFP by reverse-phase HPLC. A 15 µl (300 µg) aliquot of AFP processed as protease-treated AFP, but without protease, was injected onto a C_{18} µBondapak reverse-phase column. The column was equilibrated and eluted as described in the legend to Fig. 20. Proteins eluting from the column were monitored spectrophotometrically by absorbance at 206 nm and 280 nm as displayed in **A**. Individual fractions collected over the entire run were pooled and dried under vacuum. An aliquot (2.5 µl) was analyzed by SDS-PAGE and is displayed in lane 3 of **B**. Also included in **B**: lane 1, molecular weight markers (top to bottom) are phosphorylase B (94 Kd), bovine serum albumin (67 Kd), ovalbumin (43 Kd), carbonic anhydrase (30 Kd), soybean trypsin inhibitor (20.1 Kd), and α-lactalbumin (14.4 Kd); and lane 2, AFP.


A
A206nm
A280nm
60
40
20
0
% ACETONITRILE (------)
0
10
20
30
40
50
60
65
TIME (min)

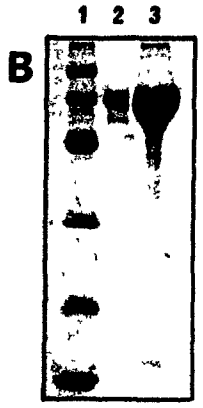
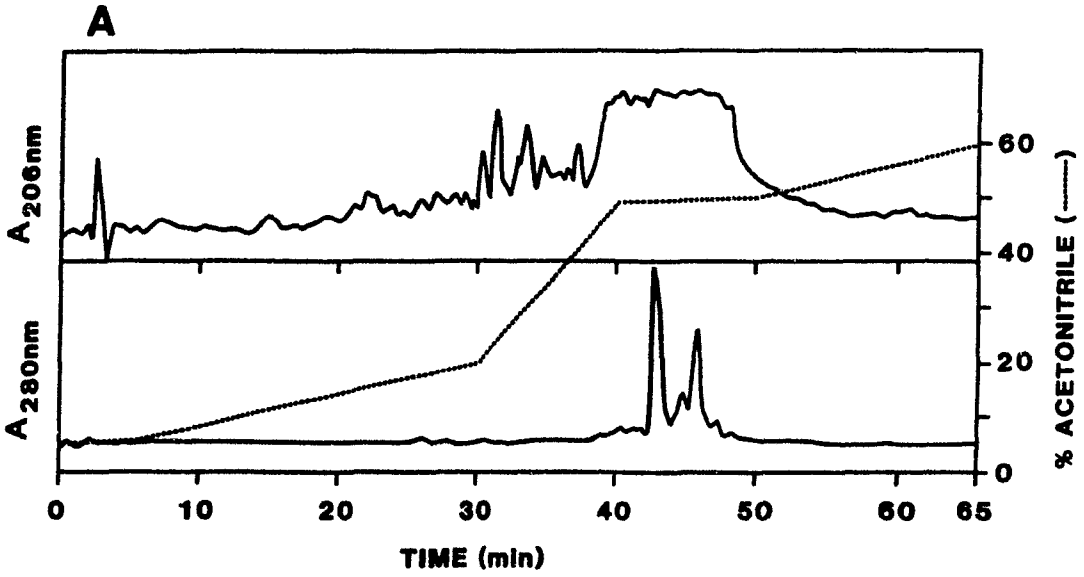

1 2 3
B

Figure 22.

Preparative chromatography of endo Glu-C-treated AFP by reverse-phase HPLC on a C_{18} μBondapak reverse-phase column. Large-scale limited proteolytic digestion of AFP by endo Glu-C was carried out as described in Materials and Methods. Two 200 μl aliquots, a total of about 8 mg, of endo Glu-C-treated AFP were injected, one immediately after the other, onto a C_{18} μBondapak reverse-phase column. The elution profile was monitored spectrophotometrically at 206 nm and 280 nm and is displayed in **A.** The column was equilibrated and eluted as described in the legend to Fig. 20. One-milliliter individual fractions were collected, dried under vacuum, resuspended with PBS and an aliquot (2.5 μl) of each fraction analyzed by SDS-PAGE using a 15% (w/v) acrylamide resolving mini-slab gel. The gel was stained with Coomassie Blue. **B** demonstrates SDS-PAGE analysis of fractions revealing partial separation of cleavage products: lane 1, molecular weight markers as described in the legend to Fig. 21B; lane 2, AFP; lane 3, endo Glu-C-treated AFP; lane 4-12, fractions eluted at 39, 40, 41, 42, 43, 44, 45, 46, and 47 mins., respectively; and lane 13, endo Glu-C.


A
A206nm
A280nm
60
40
20
0
% ACETONITRILE (——)
0
10
20
30
40
50
60
65
TIME (min)

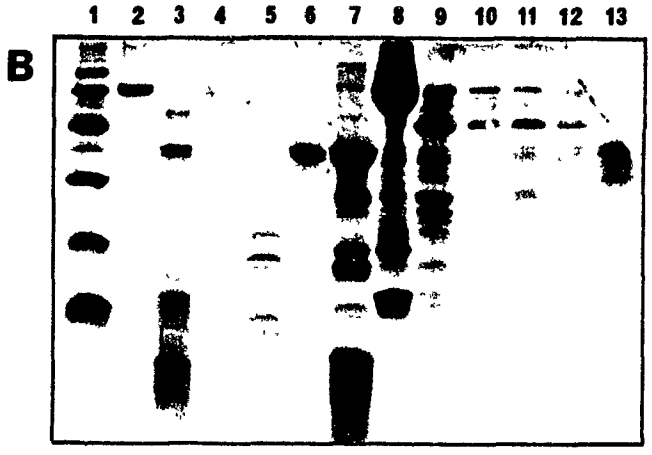
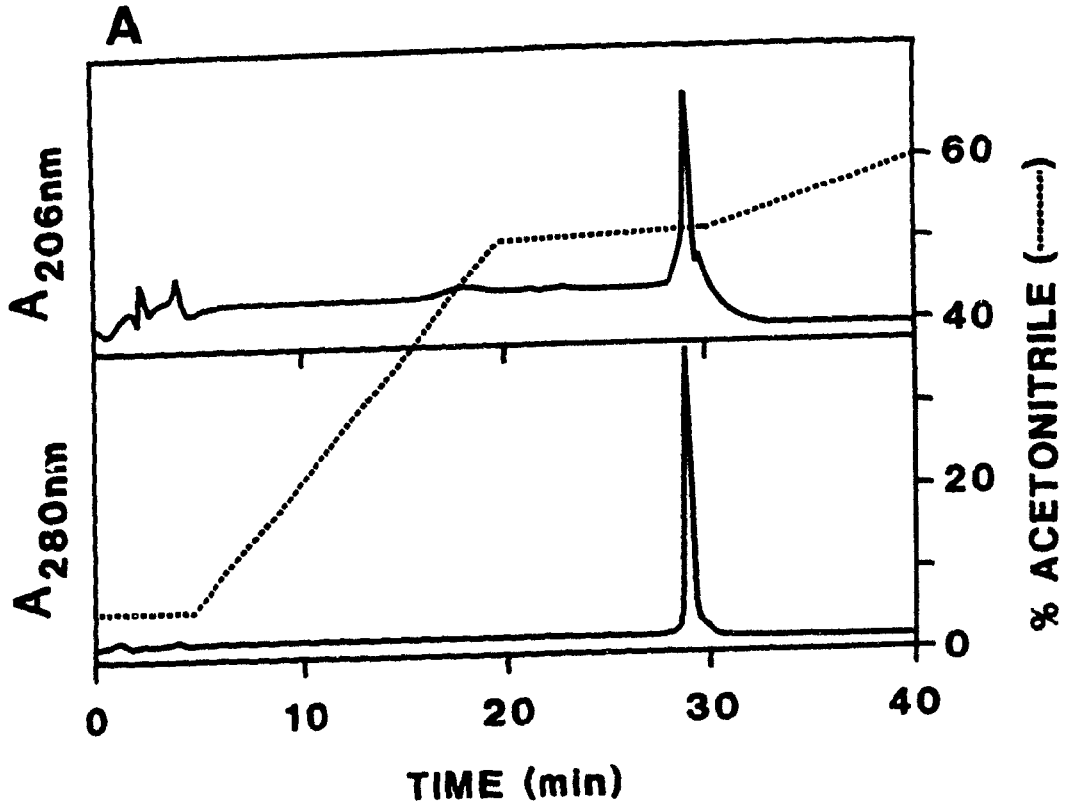

1 2 3 4 5 6 7 8 9 10 11 12 13
B

Figure 23.

Analytical rechromatography by reverse-phase HPLC of the fraction eluted at 41 mins. of the preparative chromatogaphic run of endo Glu-C-treated AFP. An aliquot (30 µl) of the fraction which eluted at 41 mins. of the preparative chromatographic run depicted in Fig. 22**A** was injected onto a C_{18} µBondapak reverse-phase column. The column was equilibrated as described in the legend to Fig. 20 and eluted in 40 mins. with an acetonitrile gradient containing 0.1% TFA as depicted in **A**, at a flow rate of 1.0 ml/min. One-milliliter individual fractions were collected and dried by vacuum for analysis by SDS-PAGE as described in the legend to Fig. 22. There is no further separation of the cleavage products of any kind as demonstrated by the SDS gel patterns displayed in **B**. **B** shows the SDS-PAGE analysis of: lane 1, molecular weight markers as described in the legend to Fig. 21**B**; lane 2, AFP; lane 3, endo Glu-C-treated AFP; lane 4, fraction eluted at 41 mins. from the preparative chromatographic run (Fig. 22**A**); lane 5-6, fractions eluted at 28 and 29 mins., respectively, of the elution profile displayed in **A**; and lane 7, endo Glu-C.


A
A206nm
A280nm
60
40
20
0
% ACETONITRILE (.....)
0
10
20
30
40
TIME (min)

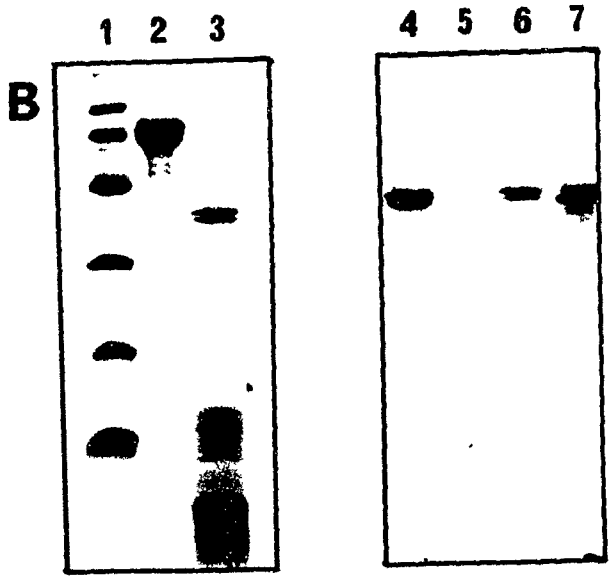
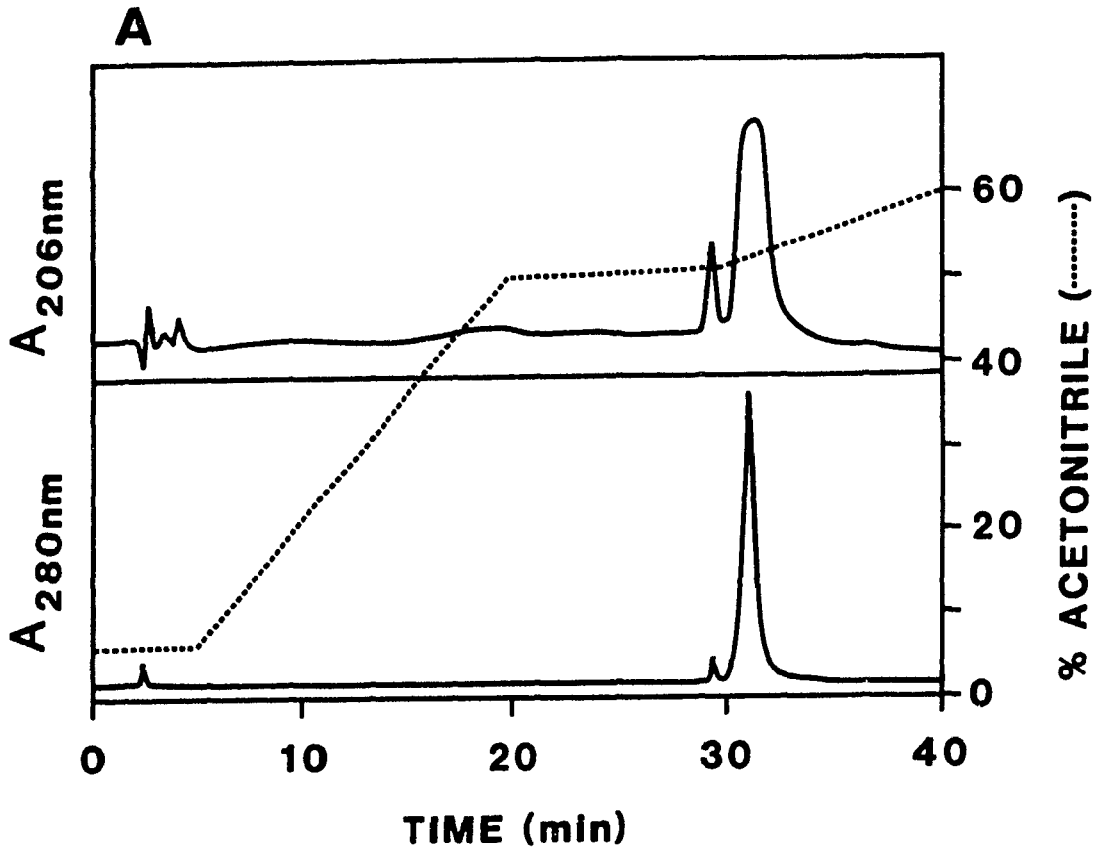

1 2 3
4 5 6 7
B

Figure 24.

Analytical rechromatography by reverse-phase HPLC of the fraction eluted at 43 mins. of the preparative chromatographic run of endo Glu-C-treated AFP. An aliquot (30 µl) of the fraction which eluted at 43 mins. of the preparative chromatographic run depicted in Fig. 22A was injected onto a C_{18} µBondapak reverse-phase column. The column was equilibrated and eluted as described in the legend to Fig. 23. Its elution profile is displayed in Fig. **A**, and individual one-milliliter fractions were collected and dried by vacuum for analysis by SDS-PAGE as described in the legend to Fig. 22. **B** demonstrates the SDS-PAGE of: lane 1, molecular weight markers as described in the legend to Fig. 21B; lane 2, AFP; lane 3, endo Glu-C-treated AFP; lane 4, fraction eluted at 43 mins. obtained from the preparative chromatograph run (Fig. 22A); lane 5-7, fractions eluted at 28, 29 and 30 mins. of the chromatograph displayed in **A**, respectively; and lane 7, endo Glu-C.


A
A206nm
A280nm
60
40
20
0
% ACETONITRILE (.....)
0
10
20
30
40
TIME (min)

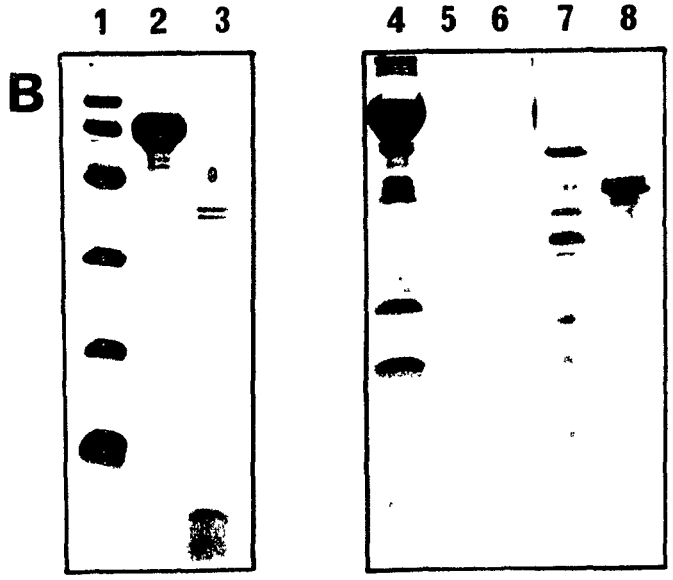
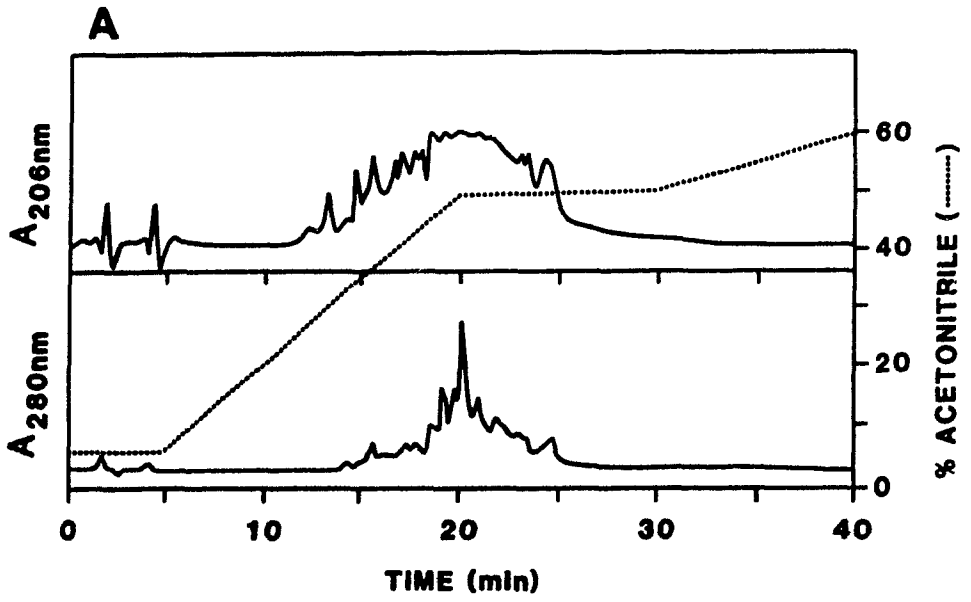

1 2 3
4 5 6 7 8
B

Figure 25.

Preparative chromatography of endo Glu-C-treated AFP by reverse-phase HPLC on a C_{18} Vydac reverse-phase column. Large-scale limited proteolysis of AFP by endo Glu-C was carried out as described in Materials and Methods. Two 200 µl aliquots, a total of about 8 mg, of endo Glu-C-treated AFP was injected onto a C_{18} Vydac reverse-phase column. The column was equilibrated as described in the legend to Fig. 20 and eluted with an acetonitrile gradient containing 0.1% TFA in 40 mins. as depicted in **A**, at a flow rate 3.0 ml/min. One-milliliter individual fractions were collected and dried under vacuum for SDS-PAGE analysis as described in the legend to Fig. 22. **B** and **C** demonstrate SDS-PAGE analysis of fractions revealing partial separation of cleavage products. Both in **B** and **C**: lane 1, molecular weight markers as described in the legend of Fig. 21**B**; lane 2, AFP; and lane 3, endo Glu-C-treated AFP. **B**, lane 4-13, fractions eluted at 17 mins. and 40 sec. to 20 min. and 40 sec. with a 20 sec. interval per fraction, respectively; and lane 14, endo Glu-C. **C**, lane 4-10, fractions eluted between 21 mins. to 23 mins. with a 20 sec. interval per fraction, respectively; and lane 11, endo Glu-C.


A
A206nm
A280nm
60
40
20
0
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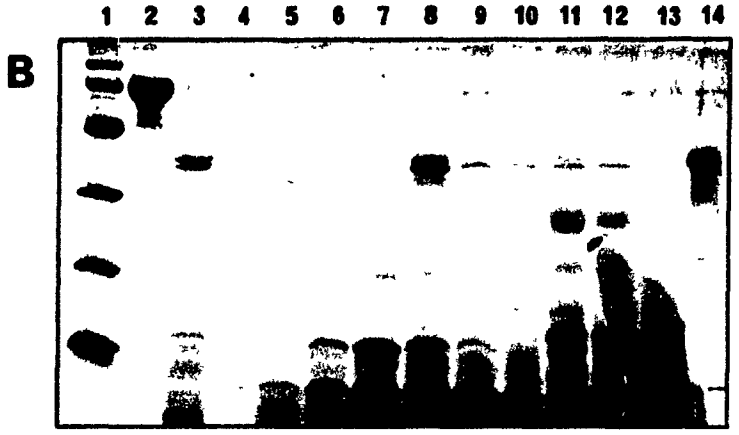
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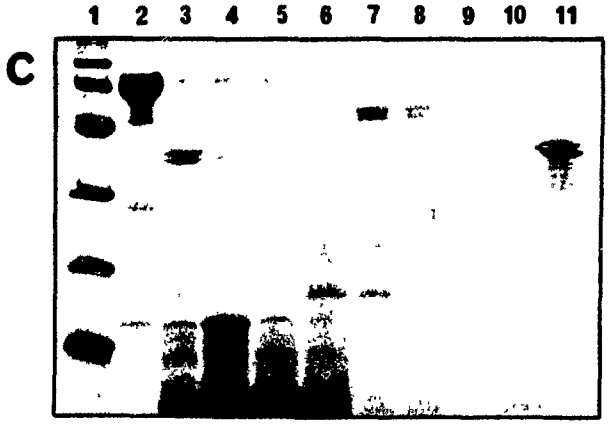
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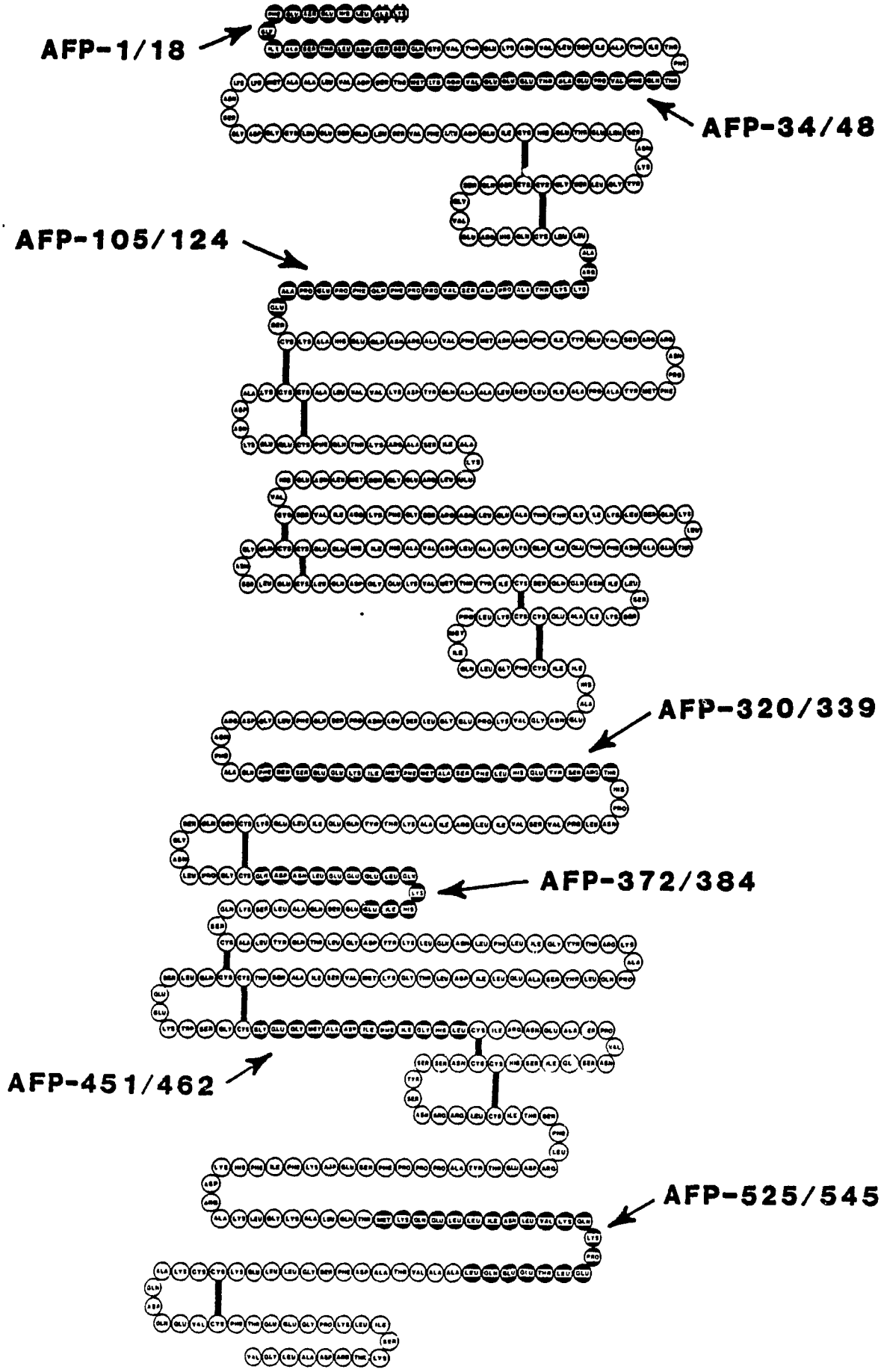
Figure 26.

The amino acid sequence of mouse AFP and synthetic AFP peptides formed into the secondary structure model developed for albumin. The amino acid sequence of mouse AFP (see reference 21) with the seven synthetic AFP peptides are, as indicated, in the secondary structure model proposed by Brown, developed for mammalian albumin. This model is based on the regularity of doublet cysteine-cysteine disulfide bridges. The synthetic AFP peptides (amino acid circles blackened in above and below) are denoted by their amino-terminal (first number) and their carboxyl-terminal (second number) corresponding to a particular peptide sequence of the parent AFP molecule.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AFP-34/48
AFP-105/124
AFP-320/339
AFP-372/384
AFP-451/462
AFP-525/545

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Discussion

Alpha-fetoprotein is a well characterized onco-fetal protein with respect to its physicochemical properties, its normal and pathological distribution, and its diagnostic value. The possible biological functions of AFP that have been suggested include (i) serving as a plasma carrier molecule for certain endogenous and exogenous substances (6,46-49); (ii) maintaining fetal osmotic pressure (6,65); and (iii) regulating the protein synthesis in the fetal stage (66). In addition, a considerable amount of evidence indicates that another important function of AFP is to exert strong and highly selective immunoregulatory effects on immune systems in vitro (30,54-58). These effects are likely to reflect important naturally occurring regulatory pathways that are particularly active in maintaining the maternal-fetal immunological relationship. However, there remains to be investigated the molecular structure-function relationship of AFP.

A considerable amount of molecular heterogeneity has been identified in both rodent- and human-derived AFP preparations. This is of potential functional relevance as different biological activity may be associated with particular molecular variants of the protein. One or more of the following factors contribute to the microheterogeneity of AFP and may be correlated with the immunoregulatory property of AFP: (i) amino acid substitution, (ii) post-synthetic modifications, (iii) variable usage of glycosylatic sites, and (iv) conformation changes.

Some studies have demonstrated correlations of the quantity of individual variants of human AFP with various physical and pathological conditions, and with differences in biological activity (61-62,105,108). The relationship between human AFP immunosuppressive potency and its origin has been investigated. These studies include AFP isolated from ascitic

fluid, serum, and liver extracts of hepatoma patients and also from serum and liver extracts of fetuses. Yachnin and Lester (71) demonstrated human AFP isolated from the serum and ascitic fluid of hepatoma patients showed a wide range of suppressive potency. The suppressive potency of AFP from human fetal liver was observed to be 1-3 orders of magnitude greater than that of tumor-derived AFP (71,76,78). Variation in biological activity was also observed in hepatoma AFPs as a class (79,109) and even among AFPs derived from serum, ascitic fluid and saline extract of tumour from a single patient with a hepatoma (109). Lester et al. (62,79,105,183) investigated the charge differences of human AFP isolates from various sources and their immunosuppressive potency. These studies demonstrated a correlation between the immunosuppressive activity of human AFP isolates from certain sources and their proportion of particular AFP species (62,183). The immunosuppressive potency of each of the human AFP isolates studied correlated with their content of the most negatively charged species of AFP (called HAFP 3) (62,183). Human AFP derived from fetal liver or tumor has a high content of HAFP 3, and is highly immunosuppressive (62). Intermediate to low levels of immunosuppresive activity are associated with serum- and ascitic fluid-derived hepatoma AFPs, respectively, and each contain a low proportion of HAFP 3 and a high proportion of the electropositive species (62,183).

The sialic acid portion of AFP may be of importance for the immunoregulatory activity of AFP. Studies carried out in the murine system with desialyated mouse AFP demonstrated the loss of the immunosuppressive activity of mouse AFP (70). However, studies by Lester et al (62) demonstrated that desialyation of human AFP did not alter its immunosuppressive activity. In addition, desialyation of human AFP did not result in the complete loss of the charge heterogeneity of the molecule

(71). They therefore concluded that the charge heterogeneity of human AFP was only partially determined by the sialic acid content of the AFP molecule (62). Also, it was speculated that the charge heterogeneity of human AFP may also be partially dependent on the primary structure of the molecule (62,63). Lester et al (109) have further suggested that post-synthetic modifications, rather than differences in the sialic acid residues present in human AFP, determines its immunosuppressive activity.

The structure-function relationship of AFP has only been partially investigated. Studies pertaining to this area of work are of extreme importance in order to achieve a better understanding of the biological significance of AFP for both theoretical and practical implications. The approach we have begun to take for the analysis of AFP's structure-function relationship is one which has already been utilized in the analysis of albumin's structure-function relationship, since AFP and albumin possess many physicochemical similarities. The structure-function relationship of albumin involved the use of fragments of BSA produced by controlled degradation. This was accomplished by limited proteolysis of the native BSA molecule. The proteolytic fragments of BSA were utilized to determine the location and properties of ligand-binding sites as well as to determine other structural, functional, and immunological features of the parent molecule.

The purpose of the present study was (i) to generate fragments of mouse AFP by limited proteolysis, (ii) to characterize the physicochemical and immunological properties of these proteolytic fragments by biochemical (SDS-, alkaline- and IEF-PAGE) and immunological (Ouchterlony immunodiffusion, immunoelectrophoresis and immunoblotting) techniques, and (iii) to isolate peptide fragments from proteolytic digestions of mouse AFP.

The method chosen for the controlled degradation of mouse AFP was by proteolytic enzymes. This method of cleavage was chosen over chemical degradation since fragments obtained by proteolysis or limited proteolysis undergo minimal chemical damage in that they have intact disulfide bonds and unaltered amino acid side chain groups (43,139). Many chemical cleavage procedures, in contrast, result in poor yields of peptide bond fission and undesirable alterations in side chain groups (138). Thus, proteolytic enzyme-derived fragments resemble more closely the native molecule than fragments obtained by chemical cleavage procedures. The proteolytic fragments, retaining many of the structural features of the native molecule, are likely to be of use in structure-function analysis of the parent molecule.

In initial studies, the optimum conditions for the proteolytic digestion of mouse AFP had to be determined. A protocol utilizing only microgram quantities of the protein, due to its limited availability, was developed. The parameters considered were pH, E:S ratio, buffer, total incubation period and even choice of protease to be used. The cleavage products generated from proteolysis of AFP with each of the proteases listed in Table 1 and under the different sets of conditions outlined in Table 2 which included various non-denaturing and denaturing (contains 0.1% [w/v] SDS) buffers, pH, E:S ratio and incubation period, were analyzed by SDS-PAGE. Protein and peptide bands were visualized in gels by Coomassie Blue staining. The pattern of peptide bands generated from these studies were found to be characteristic of the proteolytic enzyme utilized and proved to be highly reproducible. Cleveland et al. (187) made similar observations and in addition, found that the pattern of peptide bands generated is also characteristic of the protein substrate utilized.

We observed that variation of the total incubation period had no significant effect on the digestion pattern produced by the proteolysis of AFP with the various proteases utilized. We also found that in most cases, variation in the E:S ratio caused differences in the intensity of the protein band corresponding to remaining undigested protein and in the intensity of peptide bands of larger peptides. However, the majority of the peptide bands remained unchanged. The observations mentioned above coincide well with those noted by Cleveland et al. (187). In addition we and others have observed the stability of peptide patterns generated by proteolytic digestion of a protein substrate for a given protease with up to 10-fold changes in protease concentration (188). Cleveland et al. (187) demonstrated that proteolytic digestion of protein substrates in the presence of SDS resulted in the production of many relatively large peptide fragments which may be separated on high concentration acrylamide gels by SDS-PAGE. We have obtained proteolytic fragments which were separable on a 15% (w/v) acrylamide SDS gel. These fragments were also generated in denaturing (SDS-containing) buffers. In addition, for each given protease and set of conditions, we made comparisons of the digestion patterns produced by subjecting AFP to limited proteolysis carried out in the denaturing buffer versus the non-denaturing buffer. The digestion patterns generated were found to be different from one another. We found that more of the protein was digested in the denaturing buffer, but, in general, larger peptide fragments and a wider molecular weight range of peptides were produced in the non-denaturing buffer. These differences may be due to the presence of SDS in the denaturing buffer which may cause unfolding of the native AFP molecule, permitting to be exposed potential proteolytic cleavage sites which would not normally be accessible. Thus, the

availability and diversity of exposed proteolytic cleavage sites is increased. This may contribute to the observed differences in the proteolytic digestion patterns produced in the denaturing versus the non-denaturing buffers.

The limited proteolytic digestions of AFP were carried out under optimum conditions determined (summarized in Table 3) in non-denaturing or in denaturing buffers. This was performed with each protease listed in Table 1 using 2 mg of AFP per each digestion (referred to as a small-scale digestion). The optimum conditions were determined by detailed analysis of the results of studies of the micro-scale limited proteolytic digestion of AFP. In the micro-scale studies, different sets of conditions for a given protease and buffer were utilized in the digestion of AFP. The set of conditions, among those studied, that permitted various sized peptide fragments and the least remaining undigested AFP was defined as optimal. The proteolytic fragments produced under optimum conditions were further analyzed by biochemical and immunological techniques.

Sodium dodecyl sulfate-PAGE analysis of the cleavage products of these proteolytic digestions demonstrated that the digestion patterns were reproducible. The peptide band patterns were similar to those observed in the micro-scale proteolytic digestions performed under the same conditions. However, in some cases there appeared to be more remaining undigested AFP in the small-scale limited proteolytic digestions than had been present in the micro-scale limited proteolytic digestions. Differences among the digestion patterns generated in the denaturing versus the non-denaturing buffers for a given protease were also observed as had been the case in the micro-scale limited proteolytic studies. Limited proteolysis performed in denaturing buffer produced smaller peptide fragments, a narrower size range

denaturing buffer produced smaller peptide fragments, a narrower size range of peptides and less remaining undigested protein than limited proteolysis in non-denaturing buffer.

The cleavage products of the proteolytic digestions, carried out under optimum conditions, were poorly resolved by APAGE and IEF-PAGE. This made analysis of the cleavage products by these techniques difficult and inconclusive. Densitometric analysis of all of these proteolytic digestions aided in the determination of their content (percentage) of remaining undigested AFP (see Table 3). Also, some of the proteolytic fragments of these protease-treated AFP solutions were found to possess antigenic properties of the parent AFP molecule, as demonstrated by immunoblotting performed with monospecific anti-MoAFP.

Analysis of the cleavage products of the limited proteolytic digestions of AFP carried out under optimum conditions as determined in non-denaturing and denaturing buffers with each of the proteases utilized, provided important and useful information. This information was used to select which of the proteolytic digestions of AFP would be employed in performing preliminary separation studies in an attempt to isolate certain peptide fragment(s) of interest.

Conventional open column gel filtration chromatography is one of the more successful techniques utilized in the isolation of large peptide fragments. This technique was used to chromatograph elastase-treated AFP on a Sephacryl S-200 column. A large-scale limited proteolytic digestion of AFP by elastase was carried out in a non-denaturing buffer, under determined optimal conditions, resulting in a mixture containing a major large peptide fragment (apparent MW 59 Kd) and smaller fragments (apparent MW range of 12-18 Kd). Therefore, gel filtration chromatography was used

as a first step in attempting to isolate certain of the smaller peptide fragments with MWs of 12-18 Kd from the larger peptide fragment (MW 59 Kd) and remaining undigested AFP.

The use of an alkaline non-dissociating buffer did not permit the isolation of the small peptide fragments from the large peptide and residual undigested AFP, but instead resulted in their co-eluting. This problem of co-elution has been reported with the isolation of proteolytic fragments of BSA (147) and is thought to be caused by fragment aggregation which occurs near pH 7. Dissociating buffers, containing SDS or DTT were used to prevent the problem of aggregation of fragments. However, these reagents tend to interfere with absorbance measurements and did not rectify the problem of fragment aggregation since cochromatography of the cleavage products and remaining undigested AFP was still observed. The reported use of low pH buffers in the separation of peptide fragment mixtures has been found to alleviate the problem of aggregation of fragments. This was demonstrated in the partial separation (on a Sephacryl S-200 column using ammonium formate pH 3.0 as the eluting buffer [see Fig. 16A]) of the small cleavage products (MW 12-18 Kd) from most of the remaining undigested protein (see Fig. 16B, lanes 4-6) elastase digest of AFP. Total protein and peptide recovery from the column was 62%. Immunological characterization of the fractions containing partially recovered separated peptide fragments demonstrated that certain of the fragments (MWs 12-14 Kd and MW 22 Kd) possessed affinity for monospecific anti-MoAFP.

Successive zone elution of elastase-treated AFP by preparative SDS-polyacrylamide gel disc electrophoresis was performed since this digest produces proteolytic fragments of distinct MWs (ranging from 14-59 Kd) and this technique separates on the basis of MW differences. This permitted the separation of proteolytic fragments with MWs of 14-22 Kd from fragments

with MWs of 34-38 Kd, and from the larger peptides (MWs of 55-59 Kd) and remaining undigested protein (Fig. 16). However, the total protein and peptide recovery was only 21%. If this preparative separation technique were to be utilized to isolate peptides in the elastase digest, certain modifications would have to be made. A modification to be considered would be to increase the period of electrophoresis, which may allow the recovery of the larger sized peptide fragments and remaining undigested AFP. This would increase the total protein and peptide yield. Furthermore, the use of a higher concentration of acrylamide in the resolving gel may improve the resolution of certain peptide fragments. Some of the recovered peptide fragments were found to react with monospecific anti-MoAFP as demonstrated by immunoblotting experiments.

The success of reverse-phase HPLC has been well documented for speed and convenience in the separation of small peptide mixtures. For each sample the optimum solvent system and gradient must be determined. The endo Glu-C digestion of AFP carried out in non-denaturing buffer under optimal conditions, generated a peptide mixture of a wide MW range (10-50 Kd). This digestion produced a greater proportion of small peptides than did the limited proteolytic digestion of AFP by elastase also in non-denaturing buffer under optimal conditions. Analytical chromatography of endo Glu-C-treated AFP by reverse-phase HPLC, performed on a C_{18} μBondapak reverse-phase analytical column with an acetonitrile gradient containing 0.1% TFA, was found to yield two major peaks when monitored spectrophotometrically monitored at a wavelength of 206 nm (Fig. 20). HPLC analysis allowed the partial separation of the smaller peptide fragments (MW range 10-22 Kd) from the other cleavage products and from the remaining undigested AFP. All of the cleavage products by the endo Glu-C digestion of AFP that were detectable by SDS-PAGE and Coomassie

Blue staining of the gel, were demonstrated to be eluted from the reverse-phase column. There are, in contrast, reported instances where some peptides or proteins are irreversibly bound to the column and are only removed by extensive washes with a series of organic solvents. It is not uncommon that a single reverse-phase HPLC step be insufficient for the resolution of certain complex peptide mixtures (154,166,189,190,191). Therefore a two-step procedure was developed for the resolution of cleavage products generated from endo Glu-C-treated AFP. The first step involved preparative chromatography, utilizing 8 mg of the endo Glu-C-treated AFP under conditions identical to those utilized in the analytical chromatography. The second step involved rechromatography of the individual peaks obtained from the preparative chromatographic step, using a similar gradient. This procedure allowed the separation of peptide fragments with MWs of 10-22 Kd from fragments with MWs of 26-50 Kd, and from residual undigested AFP. However, co-elution of these fragments with each other and of the larger fragments with remaining undigested AFP was also observed. The latter result is not unexpected with the C_{18} μBondapak reverse-phase column since this column possesses small pores and has been reported not to be very effective in the separation of the larger hydrophobic peptides (166). Thus, preparative chromatography of the endo Glu-C-treated AFP was also conducted on a C_{18} Vydac reverse-phase semi-preparative column. This was performed under similar gradient conditions to those utilized in the rechromatography of individual fractions in the two step procedure described above, and was found to permit cleavage products with MWs ranging from 10-22 Kd to be separated from those with MWs of 26-50 Kd and remaining undigested protein. This partial separation was similar to that obtained of the preparative

chromatographic step performed on the C_{18} μBondapak reverse-phase analytical column (Fig. 22). The rechromatrography of individual fractions from the semi-preparative column with a different gradient may permit further improvement in the resolution of some of the peptide fragments.

In conclusion, our attempts to isolate fragments generated by limited proteolytic digestions of AFP did not result in the isolation of any one particular peptide fragment of AFP. However, separation of some cleavage products from others and, in certain cases, from remaining undigested AFP was realized.

The use of isolated proteolytic enzyme-derived fragments from murine AFP is one way to approach the structure-function analysis of mouse AFP. We also began to undertake another approach. This involved the use of synthetic peptides. Peptides corresponding to specific amino acid sequences of the parent mouse AFP molecule were synthesized. When synthesizing a specific peptide, a few of the many factors which were considered were size (peptide purity decreases with >30 to 40 amino acids) (114), and amino acid composition (certain amino acids are unstable and chemically modified during synthesis) (192). Seven synthetic peptides (Fig. 26) ranging from 12-21 amino acids in length, were synthesized. Two different strategies were taken in selecting which peptides were to be synthesized. The first strategy involved the choice of peptides corresponding to those amino acid sequences of mouse AFP which were most homologous to that of human AFP. These included synthetic peptides denoted AFP-1/18, AFP-372/384 and AFP-451/462. The second strategy involved the selection of the most hydrophilic regions of mouse AFP. These domains were determined by the hydropathy plot of the amino acid sequence of AFP. We focused on these regions since they were most likely exposed on the outside

of the molecule and therefore constitute potential biological active site(s). Synthetic peptides representing hydrophilic regions would also be expected to be soluble in aqueous solutions. These synthetic peptides include AFP-34/48, AFP-105/124, AFP-320/339 and AFP-525/545. All of the synthetic AFP peptides were found not to react with monospecific anti-MoAFP, even when coupled to the carrier protein BSA. This is not unexpected considering the size (1.8 to 2.9 Kd) and the estimated amount (53 to 189 μg/ml) of the synthetic peptide coupled to BSA.

Some preliminary studies were performed investigating the hypothesis that the immunoregulatory activity of mouse AFP may be localized to a particular peptide sequence of the parent molecule. The experiments involved testing the effect of both synthetic AFP peptides and coupled synthetic AFP peptides to BSA on the Con-A response of Lyt $1^{+}23^{-}$ thymocyte cells (a rapid and sensitive functional assay we routinely utilize to screen the immunosuppressive activity of mouse AFP in vitro). Only the synthetic peptide denoted AFP-34/48, representative of the amino acid sequence of AFP (amino acids 34-48), demonstrated anti-proliferative activity. AFP-34/48 at a concentration of 400 μg/ml in culture suppressed the Con-A response of Lyt $1^{+}23^{-}$ thymocyte cells by as much as 67% (see Table 8). The concentration of AFP-34/48 required for the suppression observed with the synthetic peptide is approximately 8-fold greater than the concentration of isolated native mouse AFP needed to obtain the same amount of suppression in the functional assay. The anti-proliferative activity of AFP-34/48 does not appear to be the result of nonspecific toxicity of the synthetic peptide to the cells as assessed by trypan blue dye exclusion test for cell viability. Coupling CKS-17, a synthetic peptide comprised of 17 amino acids with homology to conserved regions of

the envelope proteins of both animal and human retroviruses (119,125-127), to the carrier protein BSA was previously shown to render the peptide capable of immunosuppressive activity *in vitro* (119,129-132). At concentrations of 7.5 μM, the coupled peptide was found to suppress *in vitro* the respiratory burst of human monocytes (46.5% suppression) (129), interleukin 2-induced proliferation of an interleukin 2-dependent murine T-cell line (49.6% suppression) (119), proliferative reactions of murine lymphocytes (94% suppression) or human lymphocytes (92% suppression) in mixed lymphocyte cultures (119), and immunoglobulin synthesis by B cells (up to 99.3% suppression) (130). In addition, a 6 μM dose inhibited both endogenous (33 to 67% inhibition) and activated human natural killer cell lytic activity (46 to 66% inhibition) (131). Cianciolo *et al* (119) suggested that the biological activity associated with CKS-17 when coupled might be dependent on a particular conformation that is conferred on the peptide molecule by coupling it to the carrier protein. Each of the synthetic AFP peptides which were soluble in PBS were coupled to BSA, but this did not render any of them capable of exhibiting anti-proliferative activity as monitored by the Con-A response of Lyt 1^+ 23^- thymocyte cells.

In summary, a protocol was developed for micro-scale limited proteolytic digestion of mouse AFP with various proteases. SDS-PAGE analysis of the digestion patterns enabled us to determine the optimum conditions for the limited proteolytic digestion of AFP. These studies demonstrated that the pattern of peptide bands produced by the proteolytic digestion of AFP is characteristic of the protease utilized and was highly reproducible. In addition, the digestion patterns were found to vary a great deal with the buffer utilized. Employing a denaturing (SDS-containing) buffer allowed more efficient digestion of AFP (less remaining

undigested protein) and produced smaller peptide fragments with a narrower range of sizes than obtained using a non-denaturing buffer. The scale-up of certain limited proteolytic digestions of AFP permitted the acquisition of quantitative amounts of proteolytic fragments, while at the same time conserving the peptide band patterns found to be generated in smaller digests. Some of the proteolytic fragments possessed antigenic properties of the parent AFP molecule. Partial separations of the proteolytic fragment mixtures were achieved through gel filtration chromatography, reverse-phase HPLC, and successive zone elution by preparative SDS-polyacrylamide gel disc electrophoresis. Such proteolytic fragments are of great importance since they are potential tools for use in future investigations of the structure-function analysis of mouse AFP. These investigations might include the determination of the location and properties of ligand-binding sites, and of other structural, functional and immunological features of the molecule. Functional data with respect to the immunoregulatory activity of mouse AFP may be obtained by using proteolytic fragments and synthetic AFP peptides. Such data may complement recombinant DNA work and assist one in determining which regions of the parent molecule possess immunoregulatory activity. These long term studies may have interesting implications with respect to therapeutic applications.

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