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Tammar Wallaby Plasma Protease Inhibitory (Pi) Proteins

S. D. Patterson[A], *K. Bell*[A] *and W. E. Poole*[B]

[A]Department of Physiology and Pharmacology, University of Queensland, St Lucia, Qld 4067.
[B]Division of Wildlife and Rangelands Research, CSIRO, P.O. Box 84, Lyneham, A.C.T. 2602.

Abstract

Electrophoretic examination (isoelectric focusing and polyacrylamide gel electrophoresis) of 157 plasmas from a Kangaroo Island population of tammar wallabies (*Macropus eugenii*) resulted in the identification of five putative condominant protease inhibitor alleles, F, I, M, P and S, which exhibited microheterogeneity due to variable terminal sialic acid content. The frequencies of the five alleles in this population were 0.041(F), 0.682(I), 0.194(M), 0.073(P) and 0.010(S). The proteins had isoelectric points in the pH range 3.94–4.38, M_r of 60 500 to 66 000 and were identified as protease inhibitors by their abilities to inhibit both trypsin and chymotrypsin. Protein blotting of the denatured proteins demonstrated cross reaction with antiserum to human α1-protease inhibitor.

Introduction

The human serpin (serine protease inhibitors) family (Carrell 1984) which includes α_1-antitrypsin, antithrombin III, α_1-antichymotrypsin, $C\bar{1}$ inhibitor, heparin cofactor II and α_2-antiplasmin (Bock *et al.* 1986) has been extensively studied. Protease inhibitor (Pi) systems of other mammalian species such as the mouse, rat, cattle, sheep, pigs and dogs have recently been closely investigated. In particular, both the mouse and pig have been shown to have complex Pi systems which are controlled by two multigene families (Hill *et al.* 1985) and four closely linked loci (Gahne and Juneja 1986), respectively.

As Australia possesses such a wide array of marsupials, representing a separate pattern of mammalian organization of great antiquity, we considered it appropriate to include subclass Metatheria (or Marsupialia) in our study of mammalian Pi systems. This paper is concerned with the Pi system of tammar wallaby, *Macropus eugenii.* The tammar is a small, 4.5–8.5 kg, grey-brown wallaby, once common across a broad band of south-western Australia, but now restricted to limited areas in the south-west of the continent and a number of islands, offshore from the coasts of Western and South Australia. It is hoped that this and future studies will provide information on the evolutionary aspects of this important plasma protein system.

This report details the characterization of the products of five tammar wallaby Pi alleles, F, I, M, P and S, in terms of isoelectric point, molecular mass, inhibitory spectra, terminal sialic acid content and immunological identity using the techniques of isoelectric focusing, polyacrylamide gel electrophoresis, neuraminidase treatment and protein blotting. Some of this work has been presented previously (Patterson *et al.* 1986).

Materials and Methods

Animals

Tammars were trapped at regular intervals in yards fitted with one-way gates located at Kelly Hill Conservation Park and Flinders Chase National Park, Kangaroo Island, South Australia, during a study

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of their population dynamics (Brown and Poole, unpublished). To facilitate their examination prior to their release at the point of capture, all animals were anaesthetized and a blood sample was drawn from one of the lateral caudal veins following the methods of Poole (1982; 1986). On first capture all wallabies were individually marked in one ear with an identically numbered tag and tattoo. The population sample included 81 males and 76 females.

Plasmas

Immediately on collection, the blood samples were placed on ice. The plasmas were removed following centrifugation, stored at −20°C, transported in dry ice to the mainland and finally air-freighted to the Brisbane laboratory for electrophoretic examination. The protease inhibitory proteins were examined by isoelectric focusing (IEF) and horizontal polyacrylamide gel electrophoresis (HPAGE).

Gel Electrophoresis and Isoelectric Focusing

Horizontal polyacrylamide gel electrophoresis was performed in two different gel systems: pH 4.6, 8%T and 10%T (Bell *et al.* 1984) and linear gradient gels (HPAGGE) of 4–20%T and 10–17.5%T (Pollitt and Bell 1983). Buffer concentrations, gel dimensions, method of casting and running conditions were as outlined by Pollitt and Bell (1983) and Bell *et al.* (1984). Isoelectric focusing in the pH range 3.5–6.0 was performed as described by Patterson and Bell (1986).

Isoelectric Point Determinations

The pH gradients of the IEF gels were calibrated for the pI determinations by the use of the Pharmacia Low pI Calibration Kit (pH 2.5–6.0). Calibration curves were constructed from at least four IEF experiments by measuring the distances from the cathode that the pI markers had focused and plotting these against their corresponding pI values.

Molecular Mass Determinations

Molecular mass determinations were performed in linear gradient gels of pH 7.9, 4–20%T. Filter paper strips saturated with a mixture of Pharmacia High and Low Molecular Weight Markers were applied to the gel for calibration. The distances migrated by five of the commercial marker proteins with M_r of 232 000, 140 000, 94 000, 67 000, and 43 000 were measured and the results were analysed using a program which fitted a parabola to a set of standards (Duggleby *et al.* 1981). Calibration curves were calculated from three 2D-HPAGGE experiments.

Staining

HPAGE and HPAGGE gels were stained for protein with the Coomassie brilliant blue G250-perchloric acid stain (Holbrook and Leaver 1976), whereas IEF gels were protein stained with Coomassie brilliant blue R250 (LKB application note 1802). To ensure that all the protein spots produced following neuraminidase treatment were detected, the higher sensitivity silver staining method of Shacklee and Keenan (1986) was used. Inhibitory activities of the protease inhibitory proteins to bovine trypsin (Sigma Chemical Co.) and bovine chymotrypsin (Boehringer-Mannheim) were determined by the method of Uriel and Berges (1968).

Neuraminidase Treatment

Selected plasmas were treated with neuraminidase to determine the sialic acid contents of various Pi components in a manner similar to that described by Cox (1975) and Brown (1982) with the modifications described by Patterson and Bell (1986). Neuraminidase (from *Vibrio cholerae*, EC 3.2.1.18) of 1 U/ml activity was obtained from Calbiochem-Behring.

The numbers of sialic acid residues were determined by counting the number of basic charge shifts exhibited by each of the proteins under study following 2D electrophoresis (3.5–6.0 IEF and pH 7.9, 10–17.5%T HPAGGE) of the samples taken at various time points. The last sample included some of the protein in its asialo form.

Protein Blotting

Serial electrophoretic protein blotting was performed in a manner similar to that described by Legocki and Verma (1981) and McLellan and Ramshaw (1981) in a Bio-Rad Trans-Blot cell fitted with a supercooling coil and a power supply rated to 250 V and 2.5 A (Bio-Rad model 250/2.5). Serial transfers to nitrocellulose membrane (Bio-Rad, 0.45 μm) were run in 25 mM tris, 192 mM glycine buffer (without methanol and cooled to 6°C) at 10 V/cm for 10 min each. Four transfers were successfully obtained from

one stained or unstained gradient gel under these conditions. The use of the stained gel for transfer without the prior destaining allowed evaluation of the degree of protein transfer and the evenness of transfer across the membrane. The gel was incubated in the transfer buffer for at least 1 h prior to transfer.

Immunoblotting was performed as outlined in the Bio-Rad bulletin (goat anti-rabbit IgG alkaline phosphatase conjugate kit). Following blocking with gelatin, the membrane was incubated with either rabbit anti-human α_1-antitrypsin, antithrombin III or α_1-antichymotrypsin (DAKO) for at least 6 h (1:500 dilution) at room temperature. After incubation with the alkaline phosphatase conjugated goat anti-rabbit IgG second antibody (1:3000 dilution), visualization was achieved by using the nitroblue tetrazolium/bromo-chloro-indolyl phosphate (BCIP) reaction. All steps were performed on a gently shaking orbital shaker (Heidolph model DSG 304).

Results

1D-acid (pH 4.6) HPAGE

Plasma samples from 157 tammar wallabies were subjected to acid (pH 4.6) HPAGE and stained for protein and inhibition of trypsin and chymotrypsin (Fig. 1). Staining for protein revealed patterns (anodal to albumin) varying from three to six bands. The most common pattern which consisted of two major bands and a minor anodal band was designated type I. Similar patterns migrating more anodally and cathodally were designated F and M respectively. A rarer pattern composed of bands cathodal to M was named S. The patterns overlapped with two bands of F and I comigrating, one of I and M and one of M and S. Therefore, the numbers of bands in the combined types varied according to the types involved.

No difficulty was encountered in recognising all combinations with the exception of FI. It was found necessary to apply equal quantities (10 μl) of the appropriate plasmas to distinguish types FI and II.

The proteins of all types (F, I, M and S) were strong inhibitors of both chymotrypsin and trypsin, identifying them as protease inhibitors (Pi). Confirmation of types FI and II was also obtained by staining for protease inhibition following application of equal quantities (10 μl) of the plasmas to acid HPAGE.

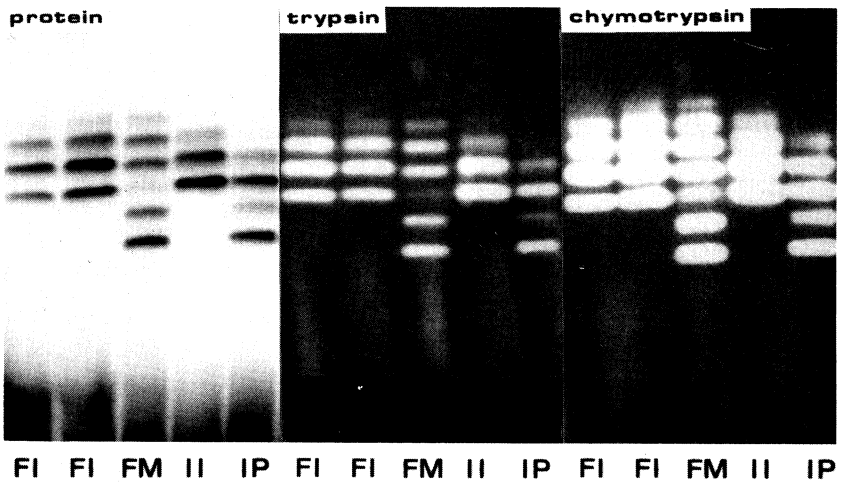

Fig. 1. Acid (pH 4.6) polyacrylamide gel patterns of tammar wallaby Pi types, FI, FM, II and IP, stained for protein and inhibition to trypsin and chymotrypsin. Only the portion of the gel anodal to albumin is shown. Pi S is not shown in this diagram. Note that Pi M and P are indistinguishable by this method. Anode is at the top.

Isoelectric Focusing

Types F, I, M and S were separable by IEF in the pH range 3.5–6.0 with patterns (three bands) similar to those described for the acid HPAGE system (Fig. 2). Exceptions were F and

I, each of which appeared to display an extra acidic band. In addition, it was possible to subdivide M into two types, designated M and P, with the latter having the more basic isoelectric points (Fig. 2).

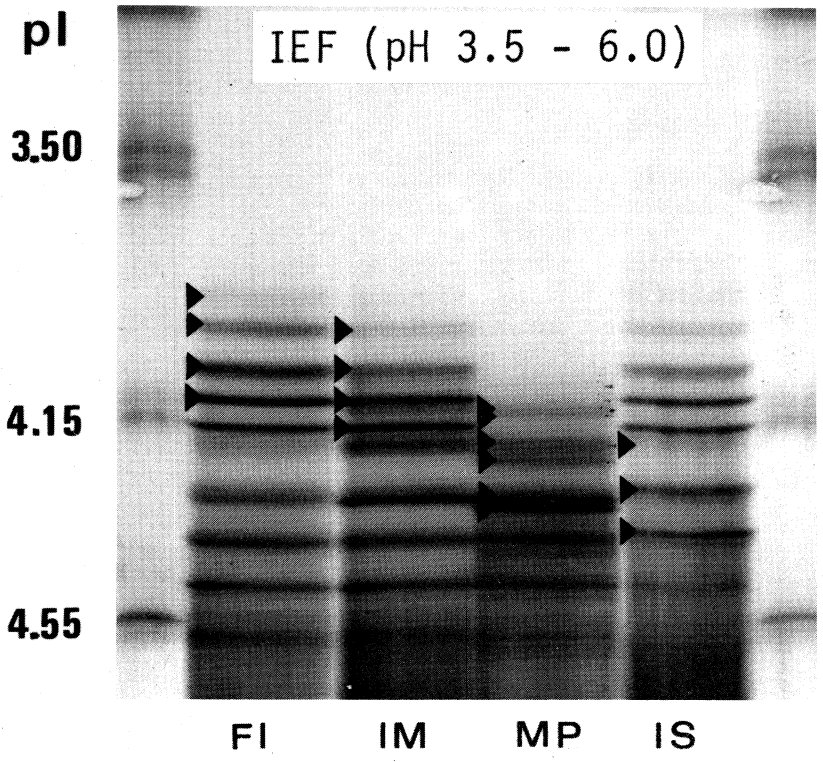

Fig. 2. Isoelectric focusing gel of four plasmas of Pi types, FI, IM, MP and IS in pH range 3.5–6.0. The gel contains the pI markers: 3.50, amyloglucosidase; 4.15, glucose oxidase; 4.55, soybean trypsin inhibitor. Arrowheads show the bands of F in FI, I in IM, M and P in MP and S in the IS samples.

The acid PAGE and IEF gel patterns of presumptive homozygotes, FF, II, MM, PP and SS, are diagrammatically represented in Fig. 3 to aid in the interpretation of actual patterns which are shown in Figs 1 and 2. The patterns for FF and SS were inferred from those of FM and IS.

ISO-DALT Electrophoresis

The ISO-DALT pattern of each Pi type consisted of two major and one minor protein spots indicating that there were no overlapping proteins in the 1D-acid HPAGE pattern. The ISO-DALT patterns together with the coordinates for each type are shown in Fig. 4. The pI and M_r ranged from pH 3.94 to 4.38 and 60 500 to 66 000, respectively. All Pi proteins inhibited both trypsin and chymotrypsin following ISO-DALT electrophoresis (Fig. 4.).

The charge shift patterns following the sequential removal of terminal sialic acid residues were visualized after silver staining. The charge shift pattern showed only one row of spots with no extra spots between the protein components (Fig. 5). This indicated that the only difference between the protein components of each type is a sialic acid residue. The three components of Pi M and P, from the most acidic to the most basic protein, appeared to contain 8, 7 and 6 sialic acid residues whereas Pi F, I and S contained 7, 6 and 5 residues respectively.

However, it was not possible to determine from the available plasmas (FM was unavailable for neuraminidase studies) which spot represented the asialo form of the Pi F protein. Hence the labelling of the second last spot with the suspected greatest number of sialic acid residues is pure conjecture as the asialo Pi F protein may be the most basic and Pi F may belong to the M and P group.

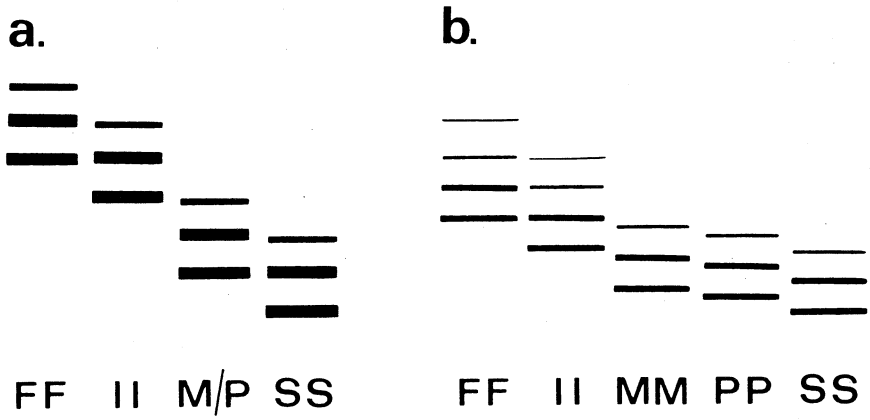

Fig. 3. Diagrammatic representations of (*a*) the acid (pH 4.6) polyacrylamide and (*b*) isoelectric focusing gel patterns of the proteins of the five homozygous types, FF, II, MM, PP and SS, found in tammar wallabies. Anode at the top.

Protein Blotting

The antisera to human α-antitrypsin showed cross-reactivity with wallaby Pi proteins when the transferred proteins had been denatured by prior protein staining (Fig. 6). However, no detectable cross reaction was found when non-stained gels were transferred. It is interesting to note that the control human plasma when denatured also reacted very strongly with the antiserum α_1-Pi. No cross reactions in the ISO-DALT patterns were observed using antisera to human antithrombin III or α_1-antichymotrypsin and transfers from stained gels.

Mating Data

The limited family data on the inheritance of the wallaby Pi variants were restricted to four dam, offspring pairs: IM, FI, II and II dams producing IM, II, II and IM offspring, respectively. These data and the electrophoretic patterns indicate that the Pi variants are controlled by five codominant alleles. Distribution of the Pi types and frequencies of the putative alleles are presented in Table 1. There were no marked differences in the gene frequencies between males and females. The Kangaroo Island population appeared to be Hardy–Weinberg equilibrium (χ^2 = 6.89, 10 d.f. $P > 0.05$).

Discussion

The protease inhibitory proteins of the tammar wallaby have been shown to be strong inhibitors of both trypsin and chymotrypsin and have pI and M_r values in the range pH 3.94–4.38 and 60 500 — 66 000, respectively. These are similar to the human alpha-1 protease inhibitory (α1-Pi) proteins which were shown by gel electrophoresis to have a pI range of pH 4.54–4.74 and M_r of 56,000 (Jeppsson *et al.* 1978). Following amino acid sequencing and carbohydrate determination, the M_r of the human α_1-Pi protein was determined to be 51 000 (Carrell *et al.* 1982). Although the available family data are scanty, it would appear from the electrophoretic results that the genes controlling the tammar wallaby Pi variants are codominant alleles.

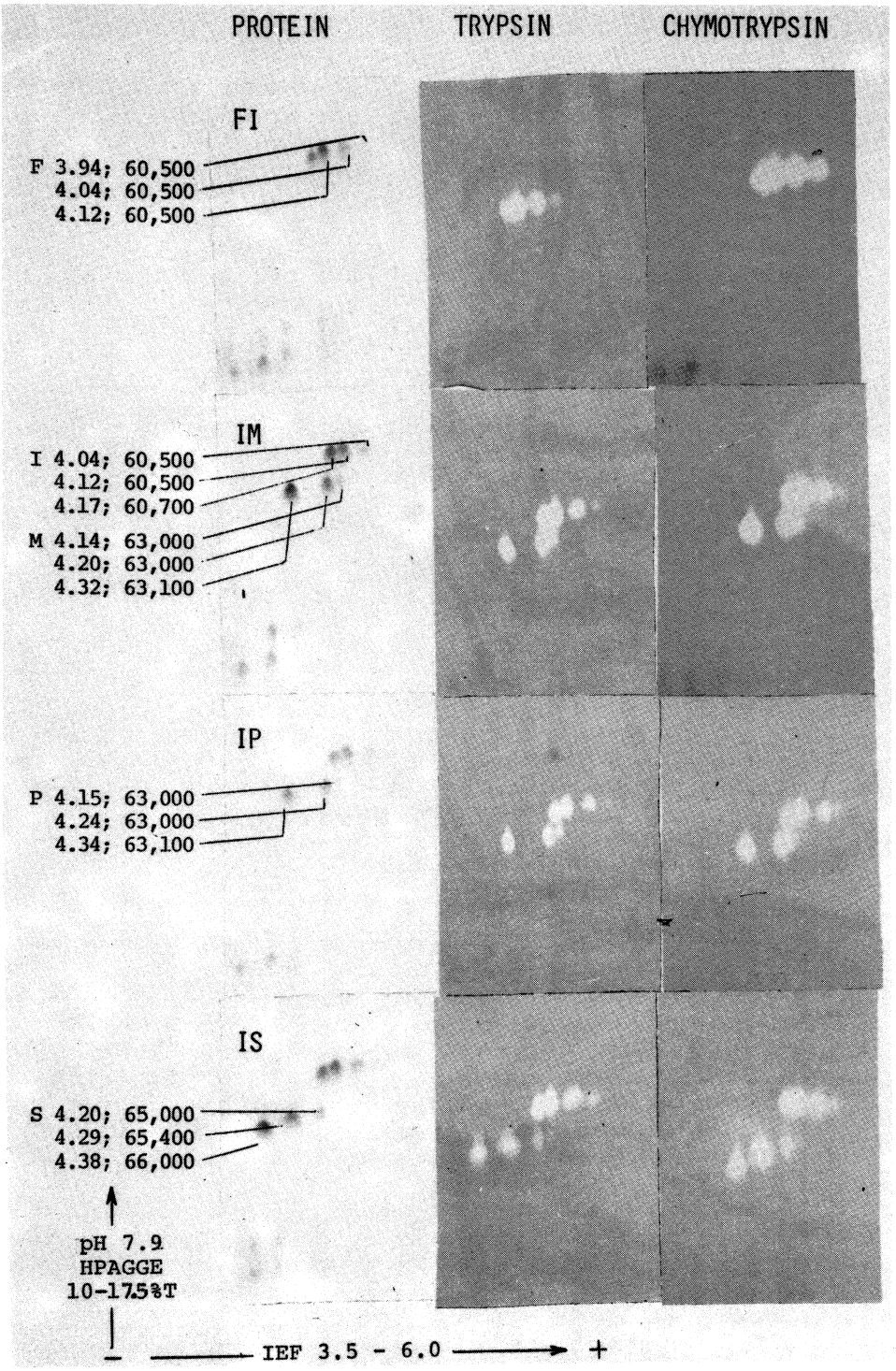

Fig. 4. ISO-DALT patterns (IEF 3.5–6.0 and pH 7.9, 10–17.5%T HPAGGE) of tammar wallaby Pi types FI, IM, IP and IS, stained for protein and inhibition to trypsin and chymotrypsin. The major proteins of each allele are labelled with their ISO-DALT coordinates.

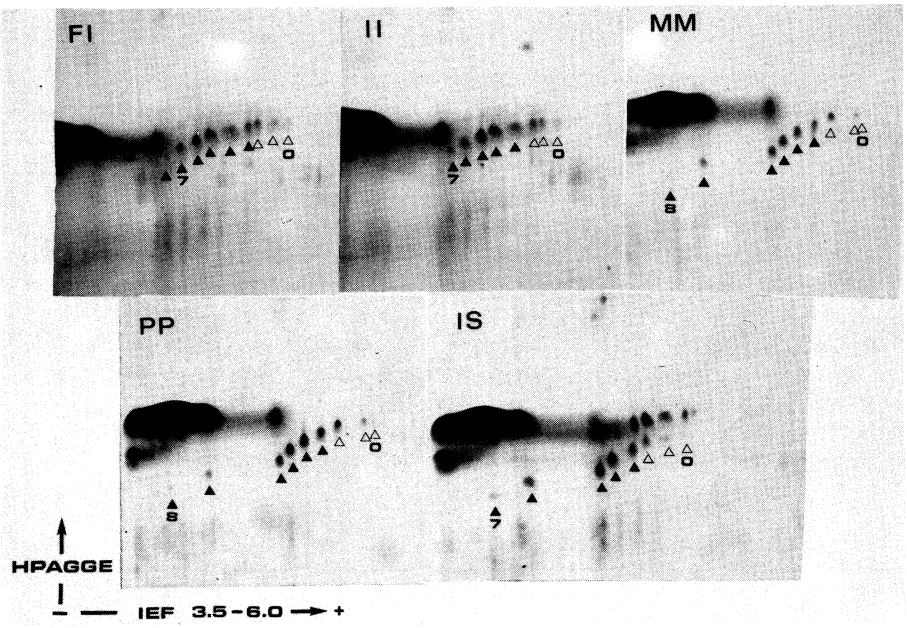

Fig. 5. Two-dimensional HPAGGE patterns (pH 3.5–6.0 and pH 7.9, 10–17.5%T) of neuraminidase-treated plasmas of Pi types FI, II, MM, PP and IS. The patterns were visualized by silver staining. Samples were mixtures of 15 min 24 h time points. Open arrowheads show the positions of the Pi proteins prior to neuraminidase treatment. Closed arrowheads show the charge shift patterns, with the numerals representing the initial and number of charge shifts to the asialo form of the protein. Acidic pI values are to be right and the anode is at the top for the second dimension.

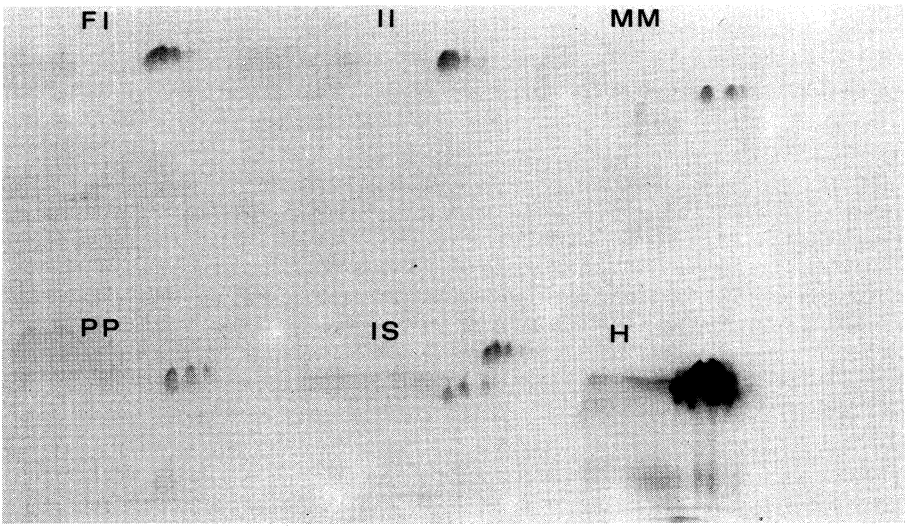

Fig. 6. Immunblots of tammar wallaby Pi types FI, II, MM, PP, IS and the human control (H) following incubation with rabbit antiserum to human α_1-Pi. The transfers were from stained 2D-gels similar to those shown in Fig. 4.

The nature of the microheterogeneity displayed by the tammar wallaby Pi alleles is predictable from the isoelectric point differences. The three components of each variant differ by 0.06–0.12 pH units (average 0.09) with no or only negligible differences in M_r. Isoelectric point differences of between 0.05 and 0.5 pH units can be interpreted as being the result of a single sialic acid residue (Beeley 1985).

This prediction appears to be confirmed by the neuraminidase treatment data. The microheterogeneity of the wallaby Pi proteins would therefore be similar to that displayed by the human α_1-Pi proteins in that both have three major isoforms that differ by one terminal sialic acid residue. The terminal sialic acid residue in human α1-Pi is attached to a branch of either a bi- or tri-antennary side chain (Vaughan and Carrell 1981). It has been shown by SDS–PAGE (Brown 1982) that the charge heterogeneity of these isoforms is accompanied by a small difference in M_r (1000) which is accountable by the mass of the additional branch which includes the sialic acid residue. The validity of the terminal sialic acid determination in this study was based on similar studies of the human α_1-Pi proteins treated with neuraminidase and examined by IEF (Jeppsson *et al.* 1978) and two-dimensional electrophoresis (Brown 1982). Structural analysis using gas–liquid chromotography subsequently confirmed these results (Vaughan and Carrell 1981). This type of microheterogeneity is thought to be caused by variable glycosylation during post-translational processing. This has also been shown to be the case for other plasma proteins such as human transferrin, which can support three different kinds of carbohydrate moieties (Marz *et al.* 1982).

Table 1. Distribution of Pi types and gene frequencies in 157 Kangaroo Island tammar wallabies

	Pi type								
	FI	II	IM	IP	IS	MM	MP	MS	PP
Number	13	70	42	17	2	7	4	1	1
	Gene frequencies								
	F		I		M		P		S
	0.041		0.682		0.194		0.073		0.010

As the Pi F charge shift pattern exactly overlaps the Pi I pattern (Fig. 5), it may be that the only difference between F and I is the degree of sialylation. This could be caused by each of the protein components of F containing an extra sialic acid residue. The apparent increase in molecular mass following neuraminidase treatment (Fig. 5) demonstrates the limitations of the non-denaturing gradient gel electrophoresis as a technique for molecular mass determinations, and hence all molecular mass values presented here are only approximate values.

Initially, IEF in the pH range 3.5–6.0 appeared to be the method of choice for Pi typing because of its ability to split Pi M into two types, M and P. However, the recent examination of additional samples from the Kangaroo Island population resulted in the detection of another Pi variant. This variant, tentatively designated J, exhibited an almost identical pattern to I on IEF (3.5–6.0) but was more readily distinguished from I on acid HPAGE. Therefore, both acid HPAGE and IEF are utilized for accurate Pi typing of tammar wallaby plasmas. The new variant, Pi J, is currently being characterized with respect to M_r, pI and sialic acid content.

The protein blotting experiments demonstrated cross reaction of the wallaby Pi proteins with antisera to human α1-antitrypsin only when denatured; these experiments suggest that the shared antigenic determinants occur at internal sites in the wallaby Pi proteins. This is in agreement with the observation of Carrell and Travis (1985) that the family of serine protease inhibitors in the human and other closely related species have approximately 30% common sequences but this homology rises to about 70% when only the hydrophobic sequences are compared. This would indicate that there has been a greater conservation of the internal (hydrophobic) residues, as would be expected if the overall tertiary structure were to be conserved.

This is the first biochemical characterization of Metatherian Pi proteins. However, the protease inhibitory proteins of most Eutherians such as cattle (Juneja and Gahne 1980*a*), sheep (Juneja and Gahne 1980*b*), rabbit (Koj *et al.* 1978), pigs (Gahne and Juneja 1986) and man (Vaughan and Carrell 1981) display the same multiple inhibitory spectra and microheterogeneity. An exception to this type of Pi microheterogeneity is that occurring in the horse (Patterson and Bell 1986).

Further study of Metatherian Pi proteins is underway. Immunological relationships within the Metatheria and between it and the Eutheria will be accompanied by protein blotting using antisera raised to the wallaby Pi proteins.

Acknowledgments

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