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A novel biochemical systematic technique for herpetology based on epidermal keratin[1]

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Summary. Using green geckos *(Phelsuma)* the molecular weights and isoelectric points of S-carboxymethylated keratin monomers from fresh and preserved reptile epidermis were investigated. Isoelectric focussing reveals that several of the circa 35 components differ between genera, species and races and that the relative similarity of the SCMK pattern is directly related to evolutionary similarity and is of considerable taxonomic value.

Biochemical systematics of reptiles and other animal groups depends largely on proteins obtained from live specimens to supply 'genetic' or molecular information. This has several disadvantages in that it is often impracticable to adequately study widely distributed species because of the difficulties in obtaining geographically comprehensive samples of live animals. It can also exclude the study of endangered, extinct and, in the case of reptiles, the many dangerously venomous species. Keratin, a structural protein, is an extremely stable polymer which can be obtained from the epidermis of preserved museum specimens as well as from freshly shed skin of reptiles. Using green geckos *(Phelsuma)* as a model, this paper shows how S-carboxymethylated components of epidermal keratin (both fresh and preserved) can be characterized by isoelectric focusing on specially formulated gels to give useful taxonomic information, particularly at the interspecific level. The use of keratin from museum specimens may overcome many of the above problems associated with the use of live specimens and thus be of considerable value to herpetological taxonomy.

The S-carboxymethylated keratin (SCMK) components are prepared in the following way. Shed skin is thoroughly washed in detergents and organic solvents and is then reduced by magnetic stirring for 12 h in thioglycollic acid, EDTA, and 8 M urea in a nitrogen atmosphere. The samples are alkylated with iodoacetic acid at pH 9.6[2]. The SCMK sample is then dialyzed against distilled water and concentrated with an Amicon multimicrofiltration unit using a Diaflo YM5 ultrafiltration membrane.

The SCMK sample could be split (before concentration) into high and low sulphur fractions by precipitating out the low sulphur fraction with zinc acetate and redissolving with sodium citrate[3].

The molecular weights of the SCMK components were determined by comparing their mobility with that of standard markers (BDH No.44262L) when run on SDS 11% polyacrylamide disc gels following the Laemmli procedure[4]. The high sulphur SCMK components show (fig. 1) 3 bands of low mol.wt (16–20 kdaltons) whilst the low sulphur components are revealed as being of more diverse mol.wt (one group at 11–20 kdaltons and another group at 32–69 kdaltons).

These SCMK components can be characterized by PAG or gradient PAG electrophoresis[5–9] but we obtained far better results for reptile keratin with isoelectric focusing (IEF).

The IEF gels were prepared from 5% acrylamide, 0.15% NN - methylene-bisacrylamide, and 8 M urea as well as 2% pH 2.5–4, 2% pH 4.0–6.0, and 1.7% pH 5.0–8.0 LKB Ampholines. The SCMK sample and both the anolyte and

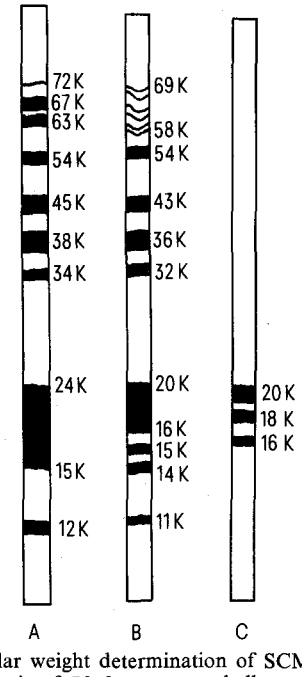

Figure 1. Molecular weight determination of SCMK components from fresh epidermis of *Phelsuma a* seychelles species. The SDS gels are drawn to scale with the computed[13] mol.wts indicated in daltons (taking into account marker dye position and gel shrinkage etc.). A, Unfractionated sample; B, low sulphur fraction; C, high sulphur fraction. Wyld and Brush[14] investigated molecular heterogeneity of keratin in a range of reptiles but do not give mol.wts for epidermal keratin of geckos or other lizards.

catholyte solutions contained 8 M urea. The gel was subjected to constant power at 30 W for 2 h. After fixing in 11.5% TCA containing 3.5% sulphosalicyclic acid the gel was stained for 3 h with 0.01% Coomassie brilliant blue R 250 IN 25% ethanol, and 10% acetic acid containing 0.1% copper sulphate and then destained in 10% ethanol and 10% acetic acid[10].

Isoelectric focusing in this manner revealed (fig. 2) that the high sulphur fraction was composed of 3 main bands with a similar isoelectric point (NB conforming to the 3 main bands in the SDS gel with similar molecular weights). The low sulphur fraction (fig. 2) contains over 20 bands of diverse pI-values conforming to the greater diversity revealed by their molecular weights.

It is apparent (fig. 2) that preservation in 70% ethanol does not affect the SCMK monomers as characterized by IEF. This is true of both high and low sulphur fractions. Consequently it should be possible to use museum specimens of reptiles for SCMK comparisons since they are generally preserved in alcohol.

A comparison of 7 *Phelsuma* species and a nocturnal gecko *(Gehyra mutilata)* (fig. 3) indicates that there are clear intergeneric differences and that each species of *Phelsuma* has its own characteristic pattern of SCMK monomers on the IEF gel.

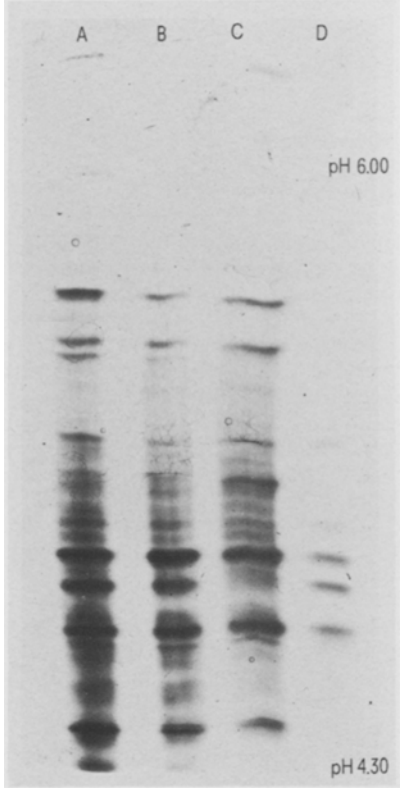

Figure 2. Isoelectric focused gel of SCMK components from *Phelsuma* seychelles *α* species. A, Unfractionated sample derived from shed skin preserved for over 6 months in 70% ethanol. B, Unfractionated sample derived from fresh shed skin. C, Low sulphur fraction from fresh shed skin. D, High sulphur fraction from fresh shed skin. Note that, concentration effects aside, there is no difference between preserved and fresh keratin (A and B). The low sulphur fraction (C) has over 20 components whilst the high sulphur fraction (D) has 3 main components, 2 of which (in this species) are isoelectrically coincident with low sulphur components.

The relative similarity of the IEF patterns can be quantified as

$$Sij = \frac{N_m}{N_p}$$

where Sij is the similarity between sample i and sample j, N_m is the number of bands in i and j with the same pI (isoelectric point) and N_p is the number of different pI-values when both sample i and j are considered.

Comparisons between relevant adjacent pairs on figure 3 show that the degree of similarity in the SCMK patterns is closely related to evolutionary similarity based on distributional criteria.

Allospecies/races (e.g. *Phelsuma α* seychelles and *β* seychelles) are very similar, S = 0.94. Closely related sympatric species, e.g. *P. ornata* and *P. cepediana* (Mauritius) are slightly less similar, S = 0.80; whilst there is even less similarity between species groups e.g. *P. α* seychelles and *P. laticauda* (Comores/Madagascar) S = 0.60. Moreover, the difference between genera e.g. *Gehyra mutilata* and *P. laticauda* is even more marked, S = 0.24.

These SMCK patterns are generally consistent within populations although 1 or 2 specific bands in particular species may be present or absent on an individual basis (e.g. *P. α*

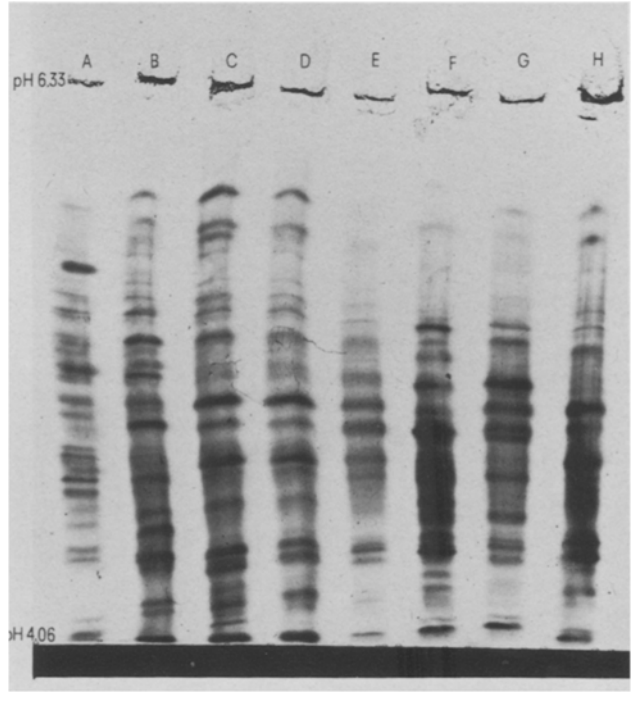

Figure 3. Isoelectric focused gel of SCMK components. A, *Gehyra mutilata*, B, *Phelsuma laticauda* (Comores); C, *Phelsuma* seychelles *α* species (Praslin); D, *Phelsuma* seychelles *β* species (Mahe), E, *Phelsuma abbotti* (Aldabra); F, *Phelsuma ornata* (Mauritius); G, *Phelsuma cepediana* (Mauritius); H, *Phelsuma astriata* (Praslin, Seychelles). E is slightly underconcentrated and F and H appear slightly overconcentrated on the photograph but were clear on the original gel.

Phelsuma α seychelles and *β* seychelles come from the Praslin and Mahe group of islands respectively. Their taxonomy is in a state of confusion the Praslin group populations being referred to as *P. madagascariensis* or *P. sunbergi* and the Mahe group populations as *P. longinsulae* or *P. abbotti.* In fact they may well be conspecific races. This is suggested by a recent field investigation of the Seychelles populations (by R.S. Thorpe) which revealed the existence of, at least superficially, intermediate forms. Since their taxonomic status is in a state of flux they are neutrally referred to as *α* and *β* seychelles species.

seychelles species a band with a pI of circa 4.45 may be present or absent in the Praslin Island population). This may be due to the influence of regulator genes but it is far more likely to be due to genetic polymorphism at the structural loci which presumably[11,12] encode for the particular keratin polypeptide. It would indeed be unusual if the genes encoding for keratin components were all monomorphic.

The number of bands differs between *Phelsuma* species. This may be due to loci being monomorphic in some species but polymorphic in others. It is, at least in part, due to different levels of isoelectric coincidence. For example, in *P. a* seychelles species 2 of the 3 main high sulphur bands are isoelectrically coincident with low sulphur bands (fig. 2). However, in *P. cepediana* there is less isoelectric coincidence between high and low sulphur bands.

Inspite of the minor loss of information due to isoelectric coincidence it is clear that there are a large number of SCMK components and they supply ample information for taxonomic and evolutionary comparisons.

Consequently, isoelectric focusing of SCMK components should provide stimulating new information for the study of evolution and taxonomy in reptiles. The wider use of isoelectric focusing, rather than PAG electrophoresis may also benefit the taxonomic usage of SCMK components in mammals and birds.

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Serotype transformation in *Paramecium primaurelia*[1]

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Summary. Transformation of 90% of the cells in a culture of *P. primaurelia* from serotype G (20 °C) to serotype D (30 °C) or vice versa was induced. The surface antigens of the 2 different serotypes have the same apparent molecular weight after reduction, and electrophoresis in SDS polyacrylamide gels, but slightly different mol.wts without reduction. Besides the specific protein with different antigenic sites for each serotype, there is at least 1 polypeptide containing the antigenic sites common to both serotype G and D, and this polypeptide probably has a lower mol.wt than the i-Ag itself.

The immobilization antigens (i-Ag) are proteins located in the surface of *Paramecium primaurelia*[3,4] and they are major components of the cell surface. They have high molecular weight[5,6] and are extractable in an aqueous solution[7]. Although the exact function of these surface proteins is unknown, the control of their synthesis is nevertheless of considerable interest to the cell biologist and geneticist. 12 different serotypes of *P. tetraurelia* have now been discovered, and they may be induced by changing the environmental conditions of the cells of a given strain. Only 1 antigen is believed to be expressed by an animal at any one time[8]. Data are consistent with the hypothesis that every serotype of the same strain has distinct antigenic proteins, the synthesis of which depends on distinct genetic loci which are nonallelic. Interstrain serotypic variations are allelic[9–11]. By using immunological techniques, workers have found that interstrain serotypes, contrary to nonallelic serotypes, have some common components.

Within a given strain of *P. primaurelia* one can find a number of clones representing different serotypes produced by temperature changes, each clone being immobilized by an appropriate dilution of homologous antiserum (antiserum directed against the same strain). Since the mechanism of the transformation process is unknown, this investigation was undertaken to further our understanding of its nature. In the work described in this paper, transformation was studied by using a biochemical technique (electrophoresis in polyacrylamide gels) simultaneously with immunochemical procedures. This method provides us with qualitative and quantitative information about antigens in different serotypes during transformation. We have been able to characterize 2 groups of proteins, which correspond to serotypes G and D, and which have different mol.wts in the presence of SDS, and in the absence of reducing agents. This difference in mol.wt allows us to follow the appearance of the new antigenic proteins.

Materials and methods. Wild type strain 168 of *Paramecium primaurelia*, which expresses serotype 168 G at 17–24 °C and serotype 168 D at 27–35 °C was used in this study. Paramecia were grown in lettuce infusion containing *Aerobacter* aerogenase according to the classical technique of Sonneborn[12]. The growth temperature was maintained at 20 °C for serotype G and 30 °C for serotype D. For serotypic transformation, a non-nutrient medium "PBS" was employed, consisting of: 7.2 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 17 mM NaCl; 2.7 mM KCl; 0.5 mM $MgCl_2$; 0.7 mM $CaCl_2$, adjusted to pH 7,2. The immobilization antigen (i-Ag) was purified by the method of Preer[7], slightly modified in that the salt alcohol extract was only precipitated by $(NH_4)_2SO_2$ at 30%, then 70% saturation. Antigen was stored in 70% saturation. Antigen was stored in 70% $(NH_4)_2SO_4$ without noticeable loss of immunological activity.