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Electrophoretic identification of fish species, or, Salmon on Friday, but Barra(on)mundi

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INFORMATION ONLY

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When fish are intact, most of the common species can be readily identified by the experienced angler, professional fisherman, wholesaler or restauranteur. Nevertheless, to identify some fish with certainty it is often necessary to call on the services of a biologist or taxonomist. When the fish has been filleted and skinned the usual morphological criteria are no longer evident and more sophisticated techniques are required. The problem of identification is more difficult if the fish has been cooked, but fortunately the technique of electrophoresis of the flesh proteins can provide the answer. One major reason for correct identification concerns misrepresentation and mislabelling either by mistake or intent. The consumer, restauranteur, retailer and wholesaler all want to be sure that they are getting what they pay for. Substitution of low priced species for high priced fish such as barramundi and John Dory has been all too common. The importer, too, wishes to ascertain that the containers he buys contain the same fish species as in the pre-purchase samples. This would be of further importance if it were a product where higher import duties apply.

For the taxonomist, electrophoresis has provided a most useful tool in distinguishing closely related species. On another note, there was a case in Italy where fillets of the toxic puffer fish were mixed in a consignment of harmless angler fish (Pocchiari 1977). Electrophoresis could have proved the identity of the toxic fillets much more readily than the complex biological assays using frogs and mice.

Because of the obvious usefulness of the technique, in 1976 the Tasmanian Food Research Unit contacted state and federal analysts and health commissions, and discovered that none of these authorities carried out such identification. Accordingly, it was decided to investigate the simplest, cheapest, most reliable technique that was applicable to the widest range of samples and

was readily usable by a variety of laboratories. This paper reports the results of the investigations.

Choice of Method

From a review of the literature the simplest method compatible with the aims of the project appeared to be that of polyacrylamide gel electrophoresis (PAGE) as described by Mackie (1969). Tube gel electrophoresis was selected rather than electrophoresis on slabs. While slabs allow for easier comparison within a single run, discs seem to afford fewer problems with diffusion and are more readily stored. Furthermore, it is much easier to compare variations from one run to the other using the discs. We have found that slabs are unwieldy, requiring gels of sufficient mechanical strength, but some of these problems can be overcome by using photographs of the results.

Principle

Much more detailed descriptions are available (Maurer 1971, Righetti *et al.*, 1979) but in essence disc gel electrophoresis involves the extraction of proteins and their application to a small column of polyacrylamide gel to which a current is applied. The proteins migrate and separate and are then 'visualised' by staining with a blue dye. A simple description of the technique has recently appeared in 'Australian Fisheries' (Anon. 1982). The pattern of bands, their thickness, density and migration distance combine to give a characteristic signature for each species (Fig. 1). In order to specify the system, all the relevant variables were investigated and the final method is described in the Appendix.

Extraction

The proteins of the sarcoplasm of fish muscle are compounds of relatively low molecular weight, many of them enzymes. They can be extracted with water

and separated by centrifugation from proteins of higher molecular weight. Extracting with water results in the extraction being done at the pH of the fish - normally 6.3 to 7.0. Buffer systems of various electrolytes are not required and distilled water is satisfactory. However shark or skate require a different treatment. In these species ammonia is produced sufficient to cause a rise in pH to about 8. The water holding capacity is then so great that the muscle takes up the extracting medium and will not release it on centrifugation. This necessitates neutralisation with acid (e.g. acetic, lactic). Myofibrillar proteins were sedimented by centrifugation at 30000xG for 30 min., but much lower forces are satisfactory. When an operator is experienced with individual species, the press juice or thaw fluids from the flesh maybe all that is required, but often the bands obtained are less discrete.

The myofibrillar proteins can be solubilised using strong urea (6-10 M) or 1-2% sodium dodecyl sulphate (SDS) solutions to dissociate hydrogen and hydrophobic bonds. These extracts must then be run on gels containing urea or SDS to keep the proteins in suspension. This results in gels with a number of bands far in excess of those required for purposes of identification, making pattern recognition quite difficult. These reagents are not interchangeable and more bands are obtained when both are used together (Laird *et al.*, 1980). When fish has been denatured by cooking, canning, pickling or even solvent extraction and subsequent drying, extraction with bond breakers is necessary to resolubilise these proteins (Fig. 2).

For canned fish, it is necessary to split proteins at methionine residues with cyanogen bromide to give protein fragments that can be separated by electrophoresis (Mackie & Taylor 1972).

Gel preparation

Standard gel preparation formulae are available, but different stocks of acrylamide must be checked to see if they have the same electrical properties. Old stocks of polyacrylamide have been found to yield gels with much higher electrical resistance. The resistance of gels changes with diameter and depth and it is important to standardise on a set tube size and a particular gel length and composition. The combination of gel length, run time, pH, and buffer strength was chosen after considerable experimentation with several widely different species of fish. This combination gave bands that were sufficiently well separated to allow recognition and identification, but were not so well separated that they were distorted, nor had they run off the end of the gel.

Run conditions

Buffers with different pH ranges are available and these can be made at different strengths. At high electrolyte concentrations there is lower resistance, heat is produced and mobility changes during the run. With the short run times employed here (approx. 20 min) and low current, the amount of heat generated was not sufficient to warrant the use of a cooling system and gels were run at ambient temperatures.

Residual persulphate ions were removed by a 15 minute pre-run before sample application. It was observed that the tracker dye band which was used to monitor run time was more discrete if the tube containing this marker was omitted from the pre-run. Each run was synchronised by switching off the current when the dye reached the end of the standard length gel. This simple criterion appears to result in summation of a wide variety of minor factors which are beyond the experimenter's control, such as degree of gel polymerisation, as well as controllable factors such as voltage, pH and ionic

strength. Thus reproducibility between runs is achieved even though run times may differ.

Sample Loading

Numerous factors affect the amount of protein that is extractable. For this reason, various amounts of extractant can be applied to the gels in order to obtain optimum loading for identification, e.g. a faint fast moving band may be the most characteristic for the species.

The mobility of the bands is not affected by loading of aqueous solutions within the prescribed limits, but where viscous urea solutions used for extracting cooked fish are run the mobility appears to vary, although the pattern is readily identifiable. Mackie (1972) has suggested addition of urea to the gels and increased buffer concentration to overcome this.

Staining and destaining

There are several stains described in the literature but amido black was found to be the quickest and simplest at both staining and destaining, although coomassie blue is said to be more sensitive. Removal of the dye using electrophoretic destaining on an open grid worked well for routine analysis of large numbers of samples and saved time.

Interpretation

The human eye is more sensitive at distinguishing faint or closely grouped bands than are scanning densitometers. Relative density can be judged by eye in a manner adequate for most purposes of identification. The photographic conditions required to reproduce the gels are set out in the Appendix but it should be noted that during printing and developing, faint bands can be lost and the most satisfactory system is to use 2¼ square contact prints. These prints are close to two-thirds actual size of the gel and there is no

loss of detail. Furthermore, they are a very convenient, concise way of storing the information. In many instances copies of gels, sufficiently good for day-to-day routine uses, can be obtained from good quality photocopying machines.

Reproducibility

The reproducibility of the method was assessed using extracts from jack mackerel. For a typical band which runs 3 cm from the origin the differences between gels in the same run from the one extract is about 1%; between different extracts from the same fish the variability is about 2%. With a band that moves only 1 cm, the variability is much higher because the errors involved in measurement are larger than the variability in the method. The general conclusion is that the reproducibility of the method can be within the limits of measurement error. However, this does not eliminate the need to have a standard authentic sample within the same run, when positive identification is required.

If, in a particular species, the proteins migrate too short a distance to give a recognisable pattern, the analysis can be repeated using a longer run time. Where two species give patterns that are difficult to distinguish, variation in run times, pH or voltage conditions can be used to help differentiation.

Factors which may change pattern

The pattern did not change for fish which had been stored on ice for three weeks by which time spoilage had gone beyond an acceptable level. Freezing the flesh does not change the pattern, which is fortunate as most samples are sent frozen to laboratories. Many fish deteriorate considerably in frozen storage and their proteins denature, particularly in species which

form formaldehyde from trimethylamine oxide. The myofibrillar proteins denature first and eventually the sarcoplasmic proteins also. This decreases the extractability and higher loadings are required on the gels. However, the electrophoretic pattern persists far beyond the point at which the fish becomes completely unmarketable (see Fig. 3).

Mixtures

Electrophoresis has proved potentially capable of resolving the components of mixtures of minced fish flesh (Podeszewski & Zarzycki 1978, Hume & Mackie 1980, Lin & Lanier 1980). This technique may assume even more importance as methods for recovery of prawn, lobster and crab meats from wastes improve and the recovered flesh is used in formulated products.

Library

A library has been set up of photographs of gels of the variety of species investigated to date. Tentative identifications can be done from this library but for absolute certainty authentic samples need to be included in an electrophoretic run. At present the simplest way to provide for this is to subdivide and store authentic samples at as low a frozen temperature as is possible. Another alternative is to freeze ampoules of the extract. Such extracts can also be freeze-dried but difficulties in rehydration have been encountered (although sonication helps) and the process is not as straightforward as was first believed. The bands from the reconstituted proteins are more diffuse and thus the patterns are more equivocal.

Markers

In the preliminary work with the densitometer bovine serum albumen was used as a marker for determination of R_f values. When visual observation was found to be sufficient the use of serum albumen was discontinued.

Other techniques

Recently, other workers (Bai & Radola 1977, Krzynowck & Wiggin 1979, Lundstrom 1979) have employed isoelectric focusing (IEF) on agarose gels for identification. Polyacrylamide gels can be used for IEF but agarose is less expensive and less potentially hazardous as acrylamide monomer is a neurotoxin (Hamilton 1982). In IEF, ampholytes are added to the gel such that under a potential difference a pH gradient is set up; added proteins then migrate to their isoelectric point. Since the isoelectric point is a fundamental property of each protein it should not change from run to run. Thus, in theory a species need only ever be run once by this system and a photograph of the result would be a permanent record of the pattern. In practice a large number of bands are obtained, more than needed for identification. Because of the extreme sensitivity of separation - which allows separation of proteins that differ in isoelectric point by only some 0.01 units of pH - the same species sometimes exhibit isomorphic enzymes which result in slightly different band patterns (Lundstrom 1981). As a result, it is still necessary to have an authentic sample in the run to verify identification. One refinement which partly overcomes this problem of gathering too much information is to use a mixture of ampholytes to expand only a portion of the pH range and to look only at this section for identification purposes (Lin & Lanier 1980). IEF provides a means of tentative identification from the library of photographs that has benefits over the simple system outlined in this paper but it does have practical difficulties and is much more expensive in both apparatus and reagents.

Quicker methods

Extracts of fish can be applied directly to a buffered cellulose support medium which acts as a bridge between the positive and negative chambers of

the apparatus containing the electrolyte. The resolution between bands is not high and the definition in the final cleared strip does not match that of acrylamide gels, but the method is very quick and relatively cheap. It is admirably suited to routine tests where the number of possible species are few and where bands are clear and well separated (Fig. 4). Further refinement of this and other techniques allows identification of specific enzymes using coupled reaction systems applied to the gels or strips to produce some identifying colour or stain. This technique is of greater use in taxonomic and genetic studies (Hadfield *et a* . 1979, Redfield & Salini 1980). Immunodiffusion using antisera has been used successfully with meats but except for some specific and/or routine instances the technique would appear to offer no advantage over electrophoresis. The preparation of the large number of antisera required could well prove to be most difficult.

Legal aspects

Electrophoretic identification is recognised as an official method by the AOAC (1975) and has been employed by the U.S. Food and Drug Administration for many years to detect mislabelling and substitution (Gutfinski 1971). Note that where legal action is a likely result great care must be taken to preserve the chain of evidence when samples are taken and passed to the testing laboratories. If such care is not taken prosecutions may lapse due to legal technicalities about the uncertainty of the origin of the sample(s) in question.

A number of closely related species are often marketed under the one common name which is recognised by consumers. As an example, one 350 g retail pack of snapper was found to contain fillets from two different species, both of which were commonly called snapper. Therefore, caution must be exercised when a prosecution is being considered.

Examples of use

Barramundi is regarded by many as Australia's premier table fish and considerable publicity has been given to the substitution of both local and imported species for it in markets, fish shops and restaurants (Figs. 5 & 6).

Sometimes the difference between species is slight, as shown in Fig. 7, where three species of leatherjackets were examined: toothbrush leatherjacket (*Penicipelta vittiger*) ♂ and ♀, six-spined leatherjacket (*Meuschenia freycineti*) and brown-striped leatherjacket (*Meuschenia australia*). There is very little difference between the brown-striped and the six-spined leatherjacket and the toothbrush leatherjacket gives almost the same pattern. Further careful work on several samples would be required to be confident of differentiation and the differences may be detectable only in the intensities of the bands rather than their mobilities. Alternative techniques such as IEF or staining for specific enzymes could confirm differences.

It had been rumored that scallop-like shapes were being punched from skate flesh and sold as scallops. This technique could clearly tell whether a suspect sample was scallop or not (Fig. 8). An importer was concerned that the shipments of Korean cod that he had bought on the basis of a satisfactory pre-purchase sample were not cod but because of their appearance and the amount of denaturation, may have been a cheaper species, Alaska pollock. The fingerprint showed that the shipments were cod and not pollock (Fig. 9), but the cod had obviously denatured in frozen storage.

Present status

Tube polyacrylamide gel electrophoresis is a relatively rapid, simple and inexpensive means of identifying species of fish using small samples of fresh, frozen, cooked, canned, dried or solvent extracted flesh. It is cheaper and simpler than IEF and more certain than electrophoresis on cellulose acetate

strips. The apparatus can be made up from Perspex by any competent laboratory technician, the chemical costs are relatively low and only a simple power pack supplying constant voltage is required. Using the techniques outlined in the Appendix it should be possible to obtain good results and to build up a satisfactory reference library.

The setting up of a national library of PAGE fingerprints was mooted at the start of the project (1977). It became evident that the amount of work involved for such a project would be considerable. Potential users of the library would have different needs and the library would have to be very comprehensive to satisfy the needs of the various interested groups. For example, the importer has different requirements from the taxonomist and regulatory authorities have different requirements from the other two. Each interested group needs to identify some facility where the appropriate tests can be done. This may be at the federal or at the state level and it may involve various types of both government and private laboratories as no one laboratory is likely to cover the potential range of applications.

Acknowledgements

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References

Anon. Electrophoresis - putting the finger on substitute fish. Aust. Fish 41(7) : 19-20, 1982.

AOAC. Acrylamide disc electrophoresis method 18.080. Official Methods of Analysis of the AOAC. 12th ed. 1975.

Bai, S. G. & Radola, B. J. Characterisation of fish sarcoplasmic proteins by thin-layer gel chromatography and thin-layer isoelectric focusing. Chem. Mikrobiol. Technol. Lebensm. 5: 33-37; 1977.

Gutfinski, K. Keeping fish labels honest. U.S. FDA Papers 5(8): 15-18; 1971.

Hadfield, A. J., Ivantsoff, V. & Johnson, P. G. Clinal variation in electrophoretic and morphological characters between two nominal species of the genus *Pseudomugil* (Pisces: Atheriniformes: Pseudomugilidae). Aust. J. Mar. Freshwater Res. 30: 375-386; 1979.

Hamilton, W. D. Fish species identification by thin layer agarose isoelectric focusing and densitometric scanning. J. Assoc. Off. Anal. Chem. 65: 119-122; 1982.

Hume, A. & Mackie, I. M. The use of electrophoresis of the water-soluble muscle proteins in the quantitative analysis of the species components of a fish mince mixture. Advances in Fish Science and Technology. Connell, J.J. ed. Fishing News Books Ltd.; Farnham. 1980; p451-455.

Krzynowck, J. & Wiggin, K. Identification of species in cooked crab meat by thin layer isoelectric focusing. J. Assoc. Off. Anal. Chem. 62: 630-636; 1979.

Laird, W. M., Mackie, I. M. & Hattula, T. Studies of the changes in the proteins of cod-frame minces during frozen storage at -15°C. Connell, J.J. ed. Fishing News Books Ltd., Farnham, 1980; p428-434.

Lin, T. S. & Lanier, T. C. Determination of species composition in minced fish blocks. Third National Technical Seminar on Mechanical Recovery and Utilization of Fish Flesh; 1980; Raleigh, Washington. National Fisheries Institute; 1980; 565-579.

Lundstrom, R. C. Fish species identification by thin layer isoelectric focusing. J. Assoc. Off. Anal. Chem. 62: 624-629; 1979.

Lundstrom, R. C. Fish species identification by isoelectric focusing: sarcoplasmic protein polymorphism in monk fish (*Lophius americanus*). J. Assoc. Off. Anal. Chem. 64: 32-37; 1981.

Mackie, I. M. Identification of fish species by a modified polyacrylamide disc electrophoresis technique. J. Assoc. Publ. Anal. 83-87; 1969.

Mackie, I. M. Some improvements in the polyacrylamide disc electrophoretic method of identifying the species of cooked fish. J. Assoc. Public Anal. 10: 18-20; 1972.

Mackie, I. M. & Taylor, T. Identification of species of heat sterilized canned fish by polyacrylamide disc electrophoresis. Analyst. 97: 609-611; 1972.

Maurer, H. R. Disc electrohoresis and related techniques of polyacrylamide gel electrophoresis. Berlin: Walter de Gruyter; 1971.

Pocchiari, F. Trade of misbranded frozen fish: medical and public health implications. Instituto Superior Sanit. Ann. 13(4): 767-772; 1977.

Podeszewski, Z. & Zarzycki, B. Identification of fish species by testing the minced-meat tissue. Nahrung 22(4): 377-384; 1978.

Redfield, J. A. & Salini, J. P. Techniques of starch-gel electrophoresis of penaeid prawn enzymes (*Penaeus* spp. and *Metapenaeus* spp.). CSIRO, Division of Fisheries and Oceanography; 1980. Report 116.

Righetti, P. G., Van Oss, C. J. & Vanderhoff, J. W. eds. Electrokinetic separation methods. Amsterdam: Elsevier/North-Holland Biomedical Press; 1979.

Appendix

Reagents Acrylamide stock solution:- acrylamide 9.5g, bis-acrylamide 0.5g made up to 100mL in distilled water.

Tris-glycine buffer (pH 8.6) stock solution:- Tris 6.0g, glycine 28.8g dissolved in distilled H_2O up to 1000 mL, diluted x 10 for electrode chambers.

Ammonium persulphate:- 1g/100g in distilled H_2O.

Tracker dye:- 1 mL of 15% glycerol + 1 mL 0.05% bromophenol blue in distilled H_2O.

Stain:- 0.1% amido black in 7% acetic acid.

Destaining solution:- methanol, acetic acid, distilled water (7:1:32).

Gel preparation (5% cross linker, 5% strength):- 25 mL acrylamide stock, 5 mL tris-glycine buffer, 4 mL NH_4persulphate, 0.04 mL tetraethylmethylene diamine, made to 50mL with distilled H_2O then filtered and degassed. Mark standard 8 cm gel tube 5 mm from top with marker and fill to mark with gel solution. Fill remaining tubes with gel solution to same level. Using a fine needle (e.g. 22 gauge) on an hypodermic syringe overlay the gels with distilled water to exclude oxygen and provide a flat meniscus. After 10-15 min gels exhibit a bluish translucence indicating that polymerisation is near completion. Cure for 1 h.

Sample preparation:- Homogenise 10g of thawed fish muscle (excluding red muscle) with 10 mL of distilled water then centrifuge at 30000xg at 2°C for 30 mins. Withdraw 2-3 mL of supernatant and dilute with an equal volume of 15% W/W glycerol.

For cooked fish:- Solubilise proteins by extracting 25g of diced sample in 50 mLs of 10M urea. Centrifuge as above and use the supernatant directly for electrophoresis.

Procedure:- Insert gel tubes except one in the apparatus and close off grommet with a glass rod. The tracker dye tube does not get a pre-run. Fill chambers with buffer solution and give gels a pre-run at 300V for 15 mins. Switch off power and empty top electrode buffer. Insert tracker dye tube and refill chamber with buffer.

Sample application:- Apply 5-20 µL of sample (50-200 µg protein) carefully through the buffer to the gel surfaces using a microsyringe. Add 20 L tracker dye to the marked tube. Switch on apparatus and run at 300V until tracker dye front has migrated to the end of the tube.

Staining:- Using a fine syringe, ream and hose the gels out of the tube into marked beakers and stain for 30 min in the dye solution. Destaining is done using several changes of solvent. This solvent can be regenerated by removing the dye with charcoal. The gels are then placed in glass tubes which are then filled with 7% acetic acid and stoppered to prevent drying out. Gels have been kept in this way for over five years.

Photography:- Photography can be difficult because of the three dimensional tube and gel, both of which are transparent. Good results can be obtained using 2¼ format Ilford Pan F developed in Perceptol. Gel tubes are placed on a smoked glass screen and lit by 500W floods, two from above at 45° and one below. Correct exposure may be calculated by using a Kodak neutral test card or by using incident light reading. When developed, contact prints suffice for reference and publication.

Legends to Figures

Fig. 1 Technique shows clear differences between five species of whiptails ([1] *Coelorhynchus fasciatus*, [2] *Coelorhynchus australia*, [3] *Coelorhynchus innotabilis*, [4] *Lepidorhynchus denticulatus* and [5] *Coelorhynchus mortoni*.)

Fig. 2 Solvent extracted and dried fish flours of (1) school shark (*Galleorhinus australis*) and (2) bronze whaler shark (*Carcharinus obscuris*), extracted with 10M urea

Fig. 3 Blue grenadier (*Macruronus novaezelandiae*) from (1) authentic fillet, from (2) badly denatured frozen stored fillet and from (3) mince frozen stored (-18°C) for four years. Note how slower moving bands have disappeared from the fingerprint of the mince and have 'faded' in the fillet which had denatured to the point of inedibility.

Fig. 4 Cellulose acetate strip gel of (1) sand flathead (*Platycephalus bassensis*) and (2) tiger flathead (*Neoplatycephalus richardsoni*).

Fig. 5 Three samples (1, 2, 3) sold as barramundi in Sydney fish market and (4) authentic barramundi (*Lates calcarifer*).

Fig. 6 Cooked samples (1, 2, 3, 4) served as barramundi in four 'prestige' restaurants (two in Sydney, two in Melbourne) and (5) cooked authentic barramundi. Sample no.2 is not barramundi. Samples extracted in 8M urea. Different loadings on gel (1) have affected mobility, but the pattern is that of barramundi.

Fig. 7 Closely related leatherjacket species in which juvenile and sexual dimorphism occurs, making taxonomy difficult. (1) Toothbrush leatherjacket (*Penicipelta vittiga*) ♂, (2) toothbrush leatherjacket ♀, (3) six spined leatherjacket (*Meuschenia freycinetti*) and brown-striped leatherjacket (*Meuschenia australis*).

Fig. 8 (1) Skate (*Raja cerva*) and (2) scallop (*Pecten alba*).

Fig. 9 Fillets from (1) Korean cod pre-purchase sample of (2) shipment labelled Korean cod and (3) Alaska pollock.

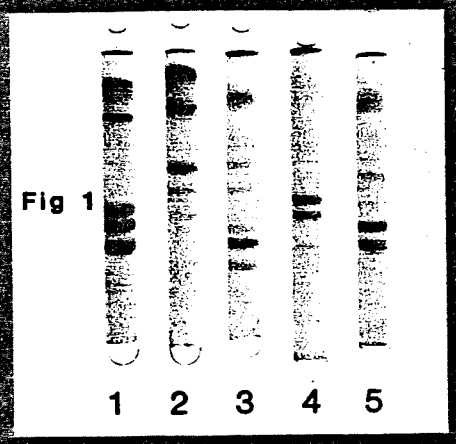
Fig 1
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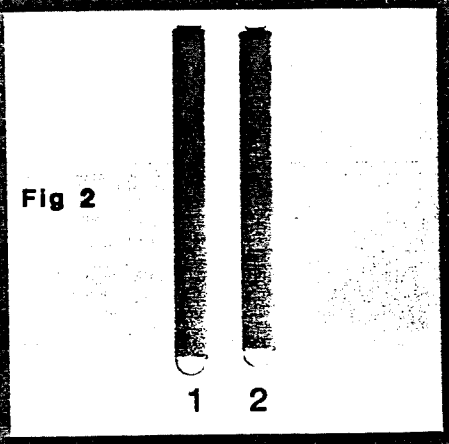
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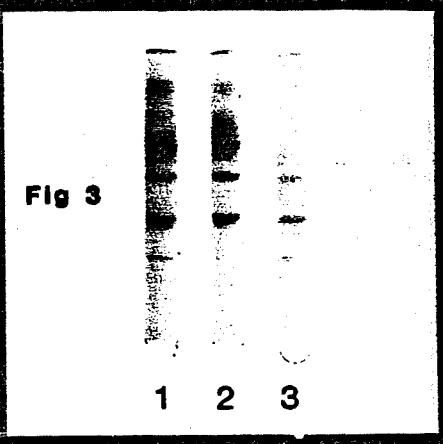
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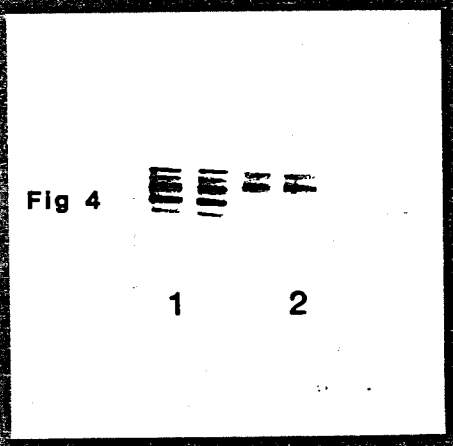
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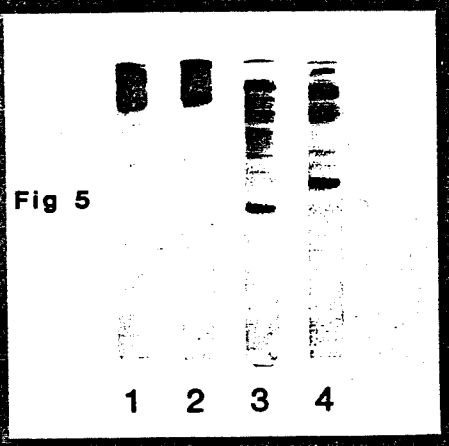
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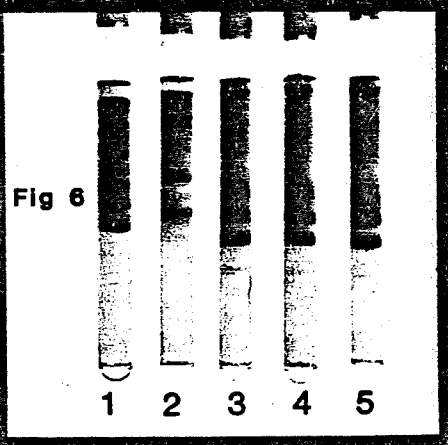
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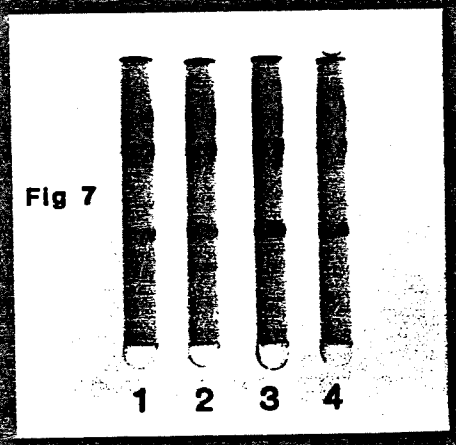
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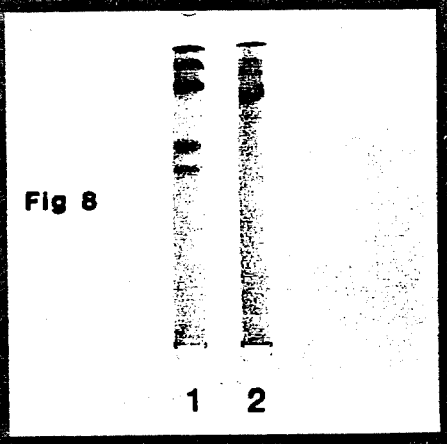
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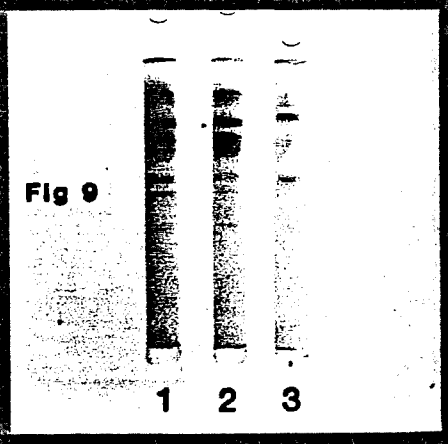
Fig 9
1 2 3