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PROGRESS REPORT ON STUDIES ON THE ESTIMATION OF GIZZEROSINE IN FISH MEAL

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SUMMARY

Two published methods for the estimation of gizzerosine in fish meal have been partially investigated. In the one method due to Maurakita *et al.* (1990) we established that the signal resulting from treatment of gizzerosine with OPA in the absence of a thiol co-reactant, unlike histidine and histamine, was exceedingly small. As this signal was utilised for the estimation of gizzerosine present in the sample the method was considered to be unlikely to offer a practicable analytical procedure. The alternative approach, as described by Ohta and co-workers, required a preliminary clean-up by ion exchange chromatography. In the absence of any specific detection reagent for gizzerosine, some variation of this approach appears mandatory. Ohta's procedure for the separation of gizzerosine from a large part of the amino acid interference has been repeated in our laboratory but HPLC analysis of the product has not yet been assessed. An ion exchange column for this purpose was purchased but problems with the flow rate of eluents together with continuing delays in the supply of synthetic gizzerosine from the CSIR have prevented completion of the trial.

Further to the above, some possible alternatives for gizzerosine analysis have been explored. Of particular interest were experiments which suggest that pre-purification of gizzerosine from other basic amino acids by ion-exchange chromatography of the N-[2,2-bis(ethoxycarbonyl)vinyl derivative might prove successful. These derivatives also form the basis of a new method of amino acid analysis by reversed phase HPLC which we have successfully employed. However, further work is necessary; current findings are encouraging.

Gizzerosine has been isolated and identified by Japanese workers as the principal toxic component of overheated protein rich materials such as fish meals. When such meals are fed to chickens in the proportions commonly used in the SA poultry industry the gizzard of the birds undergoes erosion which develops into the disease known as black vomit. Avoidance of such meals is therefore of considerable economic significance for the poultry industry.

In chemical terms, gizzerosine has been identified as 2-amino-9-(4-imidazolyl)-7-azanonic acid and is probably formed by reaction of the imidazolyl-4-ethyl group from histidine or histamine with the ϵ-amino residue of lysine. the nature of this reaction is unknown, except for the observation that overheating of the meal during production appears responsible for the toxicity. It has been established that gizzerosine is a histamine H2 receptor agonist which, *inter alia*, stimulates the production of gastic hydrochloric acid. Gizzard erosion by toxic meals can be prevented by the simultaneous administration of cimetidine, a drug known to block histamine H2 receptor sites.

From the structure of gizzerosine it is clear that the compound is an amino acid, albeit a complex one, and can be readily detected by a variety of reagents which are used to estimate amino acids. Other chemical features which may be utilised for the estimation of gizzerosine are the secondary amine residue and the imidazolyl ring. Both these features are of limited value for several reasons. Thus the imidazole ring is a very stable structure not susceptible to many detection reagents, while the small amount of secondary amine is of limited use in the presence of large amounts of primary amine. Nevertheless, consideration may have to be given to exploitation of these more specific chemical features in attempts to develop or improve analysis. In fact, the essential problem in the estimation of gizzerosine arises from the small amount of toxic principal available in the presence of very much larger amounts of other amino acids. Thus any attempt at chromatographic separation and estimation of gizzerosine is swamped by the overwhelming levels of other amino acids. In such a situation the gizzerosine peak is lost in the "noise" from these amino acids.

The initial objective of the present investigation was to assess two methods for the estimation of gizzerosine in fish meal which have been published by Japanese workers. This objective has not been met in full and further work will depend on the new administration of the Fishing Industry Research Institute, which will become a part of the CSIR in April 1995. This report will therefore summarise the present state of the investigation indicating problems still to be overcome and possible directions for future research. In both of the methods we undertook to assess, estimation of the gizzerosine was accomplished by the HPLC ion-exchange chromatography on sulphonated polystyrene resins but different techniques were employed to obtain resolution of the small gizzerosine peak. These will be reviewed later. HPLC ion-exchange columns for amino acid analyses are available from a variety of suppliers but the particular ones described in the papers we wished to evaluate were particularly expensive and beyond our budget. While it was acknowledged that the best approach was to use the same column as the original investigator it was considered acceptable to use any column manufactured specifically for amino acid analyses. For our experiments we therefore purchased a Pickering amino acid HPLC column together with a guard column.

Amino acid analyses require that both the column and post-column reaction equipment are operated at elevated temperatures and while the oven for this part of the experiment was being built a considerable length of time elapsed. It may be noted here that a column oven with temperature controller was constructed for this purpose and is working well. When preliminary HPLC experiments were eventually attempted, serious problems were experienced with the rate of solvent flow through the column. While solvents passed through the column initially at an acceptable pressure, the back-pressure progressively increased until dangerous values developed and the pumps automatically closed down. Discussions with the supplier, though helpful, have not solved the problem, and the long delay between purchase and use of the column has negated replacement by the agent. It now seems that it will be impossible to continue in this direction with the present column. Although purchase of a new column was an option, it was recognised that much recent amino acid analysis by HPLC has been carried out with reversed phase columns and we have been successful in utilising a recently published method which depends on the stable, UV detectable derivative with diethyl ethoxymethylenemalonate. Under our conditions we have succeeded in separating most of the amino acids and can readily separate gizzerosine from the basic amino acids. While this method does not have the sensitivity of a fluorescence method it has been very useful in assessing the effectiveness of a variety of chromatographic steps for the pre-purification of hydrolysates ultimately required for an effective method for gizzerosine analysis. Fluorescence methods with higher sensitivity using reversed-phase columns are available and it may be possible to adapt the method of Einarsson (1985) for secondary amino acids by formation of the Fmoc derivative after blocking of the primary amino group (in this case) with OPA. The fluorescence of the Fmoc derivatives can be measured in the presence of the OPA amino acids derivatives by the use of different parts of the spectrum. Testing this possibility may be worth while in a future study.

It must be mentioned at this stage that attempts to purchase gizzerosine have been frustratingly unsuccessful. Originally the CSIR informed us in January 1994 that they proposed synthesising gizzerosine and expected to have stocks available within six months. By September of that year they indicated that the synthesis would need another 2 to 3 months. By January 1995 we were informed that they had recently overcome a major technical problem (details not revealed) and supplies would be available in the next few months. To date we have no reliable information on when a supply will be available and the few experiments in which authentic gizzerosine has been employed have been carried out with a small sample generously supplied to the Institute by Prof Mori from Japan.

SOME CONSIDERATION ON THE MURAKITA METHOD

The method of Murakita *et al.* (1990), one of the methods we undertook to assess, depends on the selective reactivity of histidine and a few related compounds to OPA without the usually obligatory sulphydryl compound. Generally amino acids and primary amines yield a fluorescent derivative in the presence of O-phthaldialdehyde (OPA) and a sulphydryl commonly mercaptoethanol. In the absence of the thiol the method becomes virtually selective for histidine in a mixture of amino acids by virtue of an alternative reaction which yields a fluorescent product of a different, but somewhat uncertain, structure that does not require a sulphydryl residue. Histamine reacts similarly and Murakati claimed that gizzerosine can be detected in the presence of much larger amounts of other amino acids by this same reaction. As part of the present study this possibility was reinvestigated. In a comparative study it was observed that histidine did, in fact, give a large response when treated with OPA at pH 10, while histamine gave a smaller but still significant response. In our experiments gizzerosine gave only the weakest of fluorescence when treated similarly and on this observation alone the method was considered to be of no practical value. Possibly with highly sensitive fluorescence detection equipment the approach may be utilisable but it should be noted that enquiries, through the courtesy of the local Shimadzu agent, to the Analytical Laboratory of the Shimadzu Corporation, from where the method originated, indicated that the method was not yet established as suitable for routine use. Attention was therefore directed to alternative approaches.

METHODS DEPENDENT UPON PRELIMINARY PARTIAL SEPARATION OF GIZZEROSINE FROM INTERFERING AMINO ACIDS (OHTA *et al.* 1988).

It was the original intention of this study to assess the method of Ohta and co-worker (1988) in which the acid, neutral and a large proportion of the basic amino acids were removed from a sample of fish meal hydrolysate prior to HPLC on a cation exchange column. The HPLC column used was different from that specified by Marukita but it was again presumed that the Pickering column mentioned above would be applicable. Pre-purification was by low pressure column chromatography, on Amberlite CG-50 in the sodium form, from which the amino acids were eluted with sodium borate buffer pH 8,25. An interesting feature of their method is that they found the high pH resulting from $Na+$ in the effluent destroyed the gizzerosine when the sample was concentrated on the rotary evaporator. A feature of their method was therefore a second column to ensure replacement of any $Na+$ by ammonium ions prior to concentrating the eluent. Examination of their publication indicated that their separation procedure was only partially successful and was particularly ineffective in respect of arginine. The subsequent HPLC showed that gizzerosine appears in a window between extremely large peaks for arginine and lysine. We have repeated the pre-purification stage but, because of the above described problems with our ion exchange column, the effectiveness of the step has not yet been assessed. It may be noted,

however, that the pre-purification was slow and not ideal for a routine analysis. Nevertheless, in the absence of a selective method of detecting gizzerosine, such a method or some modification thereof appears the only approach. An interesting feature of the two methods considered in the present study as well as some earlier procedures is that they invariably reproduce HPLC chromatograms of fish meal hydrolysates in which the gizzerosine results from "spiking" the hydrolysate with added gizzerosine, i.e. none of the illustrations involve naturally occurring gizzerosine. We have established that gizzerosine is stable to hydrolysing conditions (acid) but have partially explored the use of enzymes for the hydrolysis. Some experiments with pepsin have been attempted but proteinase K would appear to be a better choice. Enzyme for this purpose has been purchased.

OTHER EXPERIMENTS PARTIALLY EXPLORED TO IMPROVE THE PRE-PURIFICATION OF GIZZEROSINE

Arising out of studies outlined above in which HPLC of amino acids can be achieved with the derivatives of diethyl ehtoxymethylenemalonate (DEMM) it was noted that partial separation of gizzerosine from other basic amino acids may be feasible after derivatisation with this reagent. Such a separation arises from the probable isoelectric point of each derivative considering that the DEMM modifies the primary amine residue. Thus lysine after derivatisation loses its cationic residues and should not be bound to a cationic ion exchanger. Histidine after derivatisation, by contrast, has a free carboxyl residue and an intact imidazole residue of pH approximately 6-7. The pK of such a molecule would be in the region of 5 and it should be easily eluted from a cation exchanger and separated from derivatised gizzerosine, which has a secondary amine of pK probably about 9 and imidazole residue 6-7, and arginine with its very basic guanidino residue. The pK of these two derivatives may be expected to be about 7-8. If separation cannot be achieved by selective elution with suitable eluents it was intended to modify the arginine before derivatisation by treatment with the enzyme arginase. The product of this reaction is the basic amino acid ornithine which on derivatisation with DEMM would yield an acidic product analogous to lysine. Some experiments on these lines have been carried out and Dowex 50 appears to be a suitable resin for the separation of the derivatives. Amberlite CG-50 was shown to be too weak to bind any of the derivatives though it remains suitable for separation of neutral and basic free amino acids. Further work, however, remains before the method can be established.

Finally, in an attempt to indicate alternatives to effect separation of gizzerosine from interfering amino acids, mention should be made of preliminary experiments with Ligand Exchange Chromatography (LEC), particularly on Amberlite CG-50 resin to which the metals copper or nickel have been incorporated. Resins of this type have been successfully used for amino acid analysis and separation is based on the metal-binding properties of amino acids which are frequently very different from charge, the basis of ion exchange chromatography. The method suffers from the problem that elution is almost invariably carried out with ammonia solutions which interferes with detection of the amino acids. At the time initial experiments were performed we had no suitable chromatographic procedure developed for analysis of fractions from the LEC column, but re-investigation with (say) the DEMM procedure may open up a route to a rapid pre-purification method.

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