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Aspartic acid racemization in fish meal as induced by thermal treatment

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Abstract

The effect of heat treatment during fish meal processing on amino acid racemization was studied. The hydrolysis-induced racemization rate (*R*) and the D-isomer content in the sample before hydrolysis (*I*) were differentiated by means of deuterium labelling and gas chromatography–mass spectrometry (GC–MS) analysis in selected ion monitoring mode. A preliminary experiment on laboratory-made herring meals cooked at 125°C for different time intervals showed aspartic acid (Asp) as the only amino acid with significant racemization before hydrolysis. Aspartic acid racemization rate appeared to be a nearly linear function of the duration of thermal treatment ($R^2 = 0.93$; $P < 0.01$). Analyses were carried out on several samples of commercial fish meals from different origin. Low-temperature-dried fish meals had a D-Asp content, expressed as $I = 100$ [D-isomer concentration before hydrolysis / (D- + L-isomer concentration before hydrolysis)], at less than 1%, while the D-isomer content of high-temperature dried fish meals exceeded 2%. Differences between the two commercial categories were statistically significant ($P < 0.001$). Further studies are required in order to evaluate the effects of D-Asp in protein of fish feeds and the role of the raw material and processing parameters in inducing amino acid racemization in protein of fish meals.

KEY WORDS: aspartic acid, D-amino acids in protein, fish meal quality, GC–MS, processing conditions, racemization rate.

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Introduction

The nutritional value of fish meal depends on the freshness of the raw material and on the processing conditions (Pike *et al.* 1990). Over-heating during the drying of fish meal causes complexing of certain amino acids and affects the status of cysteine/cystine residues by the formation of disulphide bonds (Opstvedt *et al.* 1984; Anderson *et al.* 1993). Bender (1972) reported that heating could cause destruction of amino acids by oxidation, modification of some of the linkages between the amino acids consequently delaying their release during digestion, and formation of linkages not hydrolysable during digestion. Carpenter *et al.* (1963) found a reduced availability of tryptophan, arginine, methionine and lysine in over-heated herring meal. As a consequence, the overall protein quality and the nutritional value of the fish meal are reduced (FAO 1986). Commercial fish meals are usually classified as 'High-temperature' or 'Low-temperature' by the producers, but, with the exception of Norway, no mandatory standards during the processing are used. Fish meals belonging to different commercial categories are characterized by substantially different prices. Therefore, a complete and detailed knowledge of their nutritional value is important. Several chemical and biological methods are reported to evaluate the quality of fish meals (March *et al.* 1966, 1985; Wilson *et al.* 1981; March & Hickling 1982; Pedersen & Eggum 1983; Hossain & Jauncey 1989; McCallum & Higgs 1989; Anderson *et al.* 1992, 1993; Romero *et al.* 1994). No data are available in the literature about the effects of heat treatment on racemization of certain amino acids within fish meal proteins. In fact, racemization of amino acids in protein chains can occur during heating and/or alkali treatment, consequently affecting the availability of certain amino acids and the digestibility of the whole protein (Liardon & Hurrel 1983). Significant activity of D-amino acid oxidase was found in the liver of several fish species, with D-alanine as a substrate (Fickeisen & Brown 1977) and free D-amino acids have been found in several marine invertebrates, but their physiological and nutritional role for aquatic animals is still unclear (Preston 1987). In fish nutrition studies, in particular, only the effects of the presence of some free D-amino acids (in particular methionine) in purified or semipurified diets have been described (Robinson *et al.* 1978; Kim *et al.* 1992), but no data are available about the effects of D-amino acids in protein.

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A preliminary study carried out at our laboratory demonstrated the occurrence of D-amino acids, particularly D-aspartic acid, in commercial fish meals (Luzzana *et al.* 1995). The aim of the present study was to verify the effects of duration of heat treatment on amino acid racemization in fish meals and to evaluate the possibility of distinguishing between 'Low-temperature' and 'High-temperature' fish meals on the basis of their D-isomer content.

Materials and methods

Samples

Five kilograms of frozen herring, *Clupea harengus* L, was obtained from commercial sources. The whole fish were minced, freeze-dried and then divided in five subsamples. One subsample was identified as the control herring meal (HFD). Following March & Hickling (1982), the four remaining samples were cooked at 127°C for 25 min, and were identified as 'Short-time-cooked' herring meal (STC). Three 'Long-time-cooked' herring meals were obtained by cooking STC at 127°C for 1 h (LTCI), 2 h (LTC2) or 3.5 h (LTC3).

A standard fish meal (SFM) and a standard bovine α-lactalbumin were obtained from Sigma Chemical Co. (St Louis, MO, USA). Three samples of 'Low-Temperature' and eight samples of 'high-temperature' commercial fish meals were obtained from commercial sources. Two 'low-temperature' fish meals were from Denmark (LTD1 and LTD2) and one from Chile (LTC). Of the 'high-temperature' samples, one was from Peru (HTP), three from Chile (HTC1, HTC2 and HTC3) and four from Denmark (HTD1, HTD2, HTD3 and HTD4).

Chemical analyses

All analyses were carried out in duplicate. Following the procedure of Bligh & Dyer (1959), 500 mg of each sample was defatted. The delipidated sample in the methanolic layer was recovered by filtration, and the extraction procedure was repeated once, to effectively remove free amino acids and soluble small peptides, as reported by Karatzas & Zarkadas (1988). The protein residue was dried overnight at room temperature and stored at –20°C until analysis. Hydrolysis was carried out following the usual precautions described by Moore & Stein (1963). Of each sample, 50mg was placed in vacuum reaction tubes (20 × 150 mm) with 10 mL deuterium chloride (^{2}HC1) 6 M containing 0.2 mL L^{-1} mercaptoethanol and 2.5 g L^{-1} phenol. Hydrolysis of bovine α-lactalbumin was carried out on 5mg, correspondingly modifying reagent volumes. Each sample was degassed several times by connecting the vacuum tube, by means of a three-way tap, to a vacuum pump and to a nitrogen cylinder. The tubes were then sealed under vacuum and placed in a ventilated oven at 110°C for 24h. After hydrolysis, the hydrochloric acid was removed under vacuum at 60°C on a rotary evaporator. The dry residue was dissolved in 20mL water and filtered on single-use filters (0.45 μm) (Sartorius AG, Göttingen, Germany): 2 mL of this solution was used for amino acid analysis. Hydrochloric acid (HCl) 0.1 M was added up to a volume of 10 mL and the sample was purified in a cation-exchange column according to Kaiser *et al.* (1974) (Dowex-50w 200–400 mesh, Sigma Chemical Co., St Louis, MO, USA). The eluate from the column was evaporated to dryness under vacuum at 60°C on a rotary evaporator and then dissolved in 3mL HCl 0.1 M. Each sample was finally evaporated to dryness under a stream of nitrogen, and the N-trifluoroacetyl-O-isopropyl esters of amino acids (N-TFA) were prepared as described by Liardon *et al.* (1981). Each sample was dissolved in 250 μL ethylacetate. The amino acid enantiomer esters were separated by capillary gas chromatography (GC) and detected by mass spectrometry (MS) in selected ion monitoring mode (SIM). The GC–MS system consisted of a model AutoSystem gas chromatograph and a model Q-Mass 910 mass spectrometer (both from Perkin Elmer, Beaconsfield, UK). The GC was equipped with a split/splitless injector and a Chirasil-L-Val 25 m × 0.25 mm capillary column (Chrompack International B V, Middelburg, The Netherlands). The injection port temperature was 220°C. The transfer line and the ion volume were heated at 200°C. Helium with a linear flow rate of 1 mL min^{-1} (inlet pressure 80 kPa) was used as carrier gas and the split ratio was 1:20. The temperature programme was from 80°C to 110°C at 3°C min^{-1}, then from 110°C to 190°C at 5°C min^{-1}, held for 9 min. Of each sample, 0.5 μL was injected. Total run time, including oven cooling, was 35 min. Before measurements were performed, the mass spectral parameters were adjusted to achieve predefinite performance criteria by tuning on *m/z* 69, 131, 219, 264, 314, 414, 502 of the tuning calibration compound perfluorotributylamine. Spectra of the N-TFA derivatives of amino acids were obtained under EI conditions at 70 eV. For quantitative SIM measurements, the mass spectrometer was adjusted to monitor 11 sets of ion masses. The signals were stored on a Digital DEC station (Digital Equipment Corporation, Maynard, MA, USA) and used to reconstruct the ion chromatograms and to measure peak area ratios. Following the method of Liardon *et al.* (1981), the D-isomers formed during the hydrolysis of fish meals were differentiated from those initially present in the samples by means of deuterium labelling. The results are expressed as D-isomer initial content I = 100 [D-isomer concentration before hydrolysis / (D-+ L-isomer concentration before hydrolysis)] and hydrolysis-induced racemization rate R = 100 (D-isomer formed during hydrolysis / remaining initial L-isomer after hydrolysis).

Statistical analysis

The relationship between D-aspartic acid initial content (I) in herring meals and duration of thermal treatment was investigated by linear regression. Differences between LT and HT commercial fish meals were tested by Student's t-test, with $P < 0.001$. All calculations were performed using the procedures of the Statistical Analysis System Institute (SAS Institute 1991).

Results and discussion

The results are reported as means of the duplicate analyses. Liardon *et al.* (1981) reported that the lower limit for a correct determination of D-isomer by the described method was 0.2–0.4%, because below this limit the determinations were impaired by the residual interference of hydrolysis-induced racemization. Therefore, we considered all the values below 0.4% as under the correct limit of detection. We separated and quantified the enantiomers of the following amino acids; alanine (Ala), valine (Val), aspartic acid (Asp), phenylalanine (Phe), glutamic acid (Glu) and lysine (Lys). Analysis of bovine α-lactalbumin standard was carried out as a preliminary experiment to evaluate the correctness of the method. Results demonstrated that the method could correctly differentiate the D-isomers formed as a consequence of hydrolysis from those originally present in the samples, obviously absent in a purified protein such as lactalbumin. On the other hand, hydrolysis-induced racemization was evident for all the six amino acids evaluated, being 0.7% for Ala, 1.7% for Val, 1.9% for Asp, 0.7% for Phe, 0.7% for Glu and 0.9% for Lys. This fact confirmed the utility of the method itself. In fact, because the degree of racemization depends not only on the amino acid, but also on its neighbours in the protein chain, the procedure of taking as blank values the racemization rates of free amino acids treated under the same conditions appears to be incorrect (Liardon *et al.* 1981). The values relative to the hydrolysis-induced racemization rate of each amino acid appeared to be similar for all the samples analysed, as expected because the hydrolysis conditions were identical for all the samples. R values ranged from 0.7% to 1.3% for Ala, from 0.5% to 0.7% for Val, from 1.4% to 3.7% for Asp, from 0.5% to 1.2% for Phe, from 0.5% to 1.0% for Glu, and from 0.5% to 0.7% for Lys. Results relative to the D-amino acid content in the herring meals before hydrolysis confirmed that aspartic acid is the amino acid most sensitive to processing-induced racemization (no significant amounts of Ala, Val, Phe, Glu and Lys D-isomers were detected), and that the percentage of this D-amino acid increased with the duration of the thermal treatment from HFD to LTC3 as shown in Fig. 1. This relationship can be described by the linear function:

$$\text{D-Asp } (\text{D} / \text{D} + \text{L } \%) = 0.13\,t + 0.40 \; (R^2 = 0.93;\ P < 0.01)$$

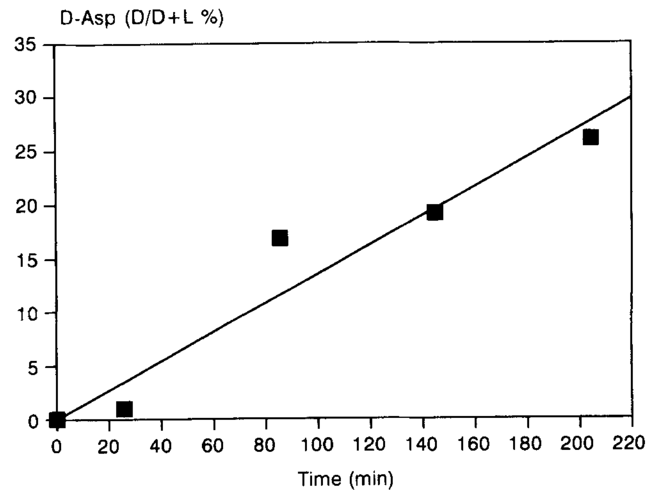

Figure 1 Relationship between D-aspartic acid content, expressed as I = 100 [D-isomer concentration before hydrolysis / (D-+ L-isomer concentration before hydrolysis)]. of laboratory-made herring meals and duration of thermal treatment at 127°C (min).

where t is duration of the thermal treatment at 127°C in mins.

Aspartic acid is not essential in fish nutrition, but the occurrence of any D-amino acid in the peptide chain could reduce the availability of its neighbours in the chain itself, because L–D, D–L and D–D peptide bonds introduced during the racemization process would resist attack by proteolytic enzymes which function best with L–L bonds. They may be, in fact, competitive inhibitors of the cleavage of L–L peptide bonds (Friedman *et al.* 1981). As a consequence, the protein digestibility and the availability of essential amino acids not subjected to racemization could also be reduced. Moreover, Friedman *et al.* (1981) also demonstrated that both lysinoalanine formation and aspartic acid racemization in casein treated at 65°C increased with treatment time, and that the extent of peptide-bond hydrolysis by trypsin followed an inverse relationship with D-isomer and lysinoalanine content. These observations, which clearly relate D-aspartic acid content with other well-known quality parameters, suggest potential application to aquaculture of D-aspartic acid content as a further quality marker of fish meal. The results relative to the D-aspartic acid content in the commercial fish meals before hydrolysis are reported in Table 1. Some differences in the D-aspartic acid content are evident within the 'High-temperature' samples (ranging from 2.0% to 5.8%). On the other hand, all the so-called 'Low-temperature' fish meals showed a D-amino acid content before hydrolysis of less than 1%, values significantly different ($P < 0.001$) from the results for the 'High-temperature' meals, which all exceeded 2% of D-aspartic acid.

Results from the present work demonstrate that heat treatments during processing of fish meals induce racemization of aspartic acid and that the processing-induced racemization appears to be a

Table 1 D-aspartic acid content, expressed as I =100 [D-isomer concentration before hydrolysis / (D-+L-isomer concentration before hydrolysis)], in commercial fish meals before hydrolysis. Values are the mean of duplicate analyses (maximum CV 15.2%) Means having different superscript letters are significantly different ($P < 0.001$).

Fish meal[1]	SFM	LTC	LTD1	LTD2	HTP	HTC1	HTC2	HTC3	HTD1	HTD2	HTD3	HTD4
D-Asp(%)	0.7^a	0.5^a	0.6^a	0.6^a	3.0^b	2.8^b	2.0^b	4.9^b	4.4^b	5.8^b	3.7^b	3.5^b

[1]SFM, standard fish meal; LTC, 'Low-temperature' fish meal from Chile; LTD1-2, Low-temperature' fish meals from Denmark; HTP, 'High-temperature' fish meal from Peru; HTC1-3, 'High-temperature' fish meals from Chile; HTD1-4, 'High-temperature' fish meals from Denmark.

nearly linear function of the duration of the thermal treatment. The D-aspartic acid content of the product could give some indications about the severity of the thermal treatment during cooking of drying, because significant differences were evident between 'High-temperature' and 'Low-temperature' fish meals. Further studies will be carried out at our laboratory, in cooperation with other research institutions, to evaluate the effects of the presence of enantiomers in feed protein on the performances of the farmed fish (i.e. eventual reduction of amino acids availability and protein digestibility together with eventual toxic effects). Moreover, some differences are evident in the D-aspartic acid content of the so-called 'High-temperature' fish meals. As a consequence, studies will also be directed towards assessing the role of the different features of the raw material (such as moisture content and pH, the latter related also to the freshness of the material used) and of the processing parameters (such as temperature, pressure and duration of the treatment) in inducing amino acid racemization in fish meals. Furthermore, because some differences in digestion coefficients for individual amino acids were observed between extruded and pelleted diets for rainbow trout (Vens-Cappell 1984), the effects of the feed process on amino acid racemization will also be studied.

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Editorial note

There have been many attempts to correlate the chemical analyses of fish meal with its nutritional quality, without any great success.

By accepting the paper by Luzzana *et al.* for publication in *Aquaculture Nutrition* we introduce a relatively new chemical method into the field of fish nutrition. While fish meal is considered as mainly a protein (amino acid) source in most terrestrial animals, it is also an important energy source for many cultured fish species. It is, therefore, of double importance that the protein is adequately digested to absorbable constituents in the digestive tract of fish. Heat treatment during fish meal production is generally accepted to negatively affect the digestibility of fish meal protein, and, therefore, some measure of digestibility is deemed to be necessary for the proper evaluation of the nutritional value of fish meal. Determination of protein digestibility with mink as the experimental animal is one alternative, another is to use 'the dilute pepsin digestibility method', which is performed *in vitro*.

The method suggested by Luzzana *et al.* purposes to determine the degree of heat treatment directly by measuring the degree of isomerisation of protein-bound aspartic acid in fish meals. The method obviously requires further refinement and also a follow up study of feeding experiments with fish. The authors have expressed their intention to do this. Therefore, they have wished that the fish nutrition society should know of the method when they later publish experiments based on its use.

The editors have found it prudent to make the method known in *Aquaculture Nutrition*. It should be stressed, however, that there are many other subtle changes, not detected in the ordinary amino acid analysis, which may occur during fish meal production. We mention the cysteine/cystine ratio, the methionine/methioninesulphoxide ratio and the aparagine+glutamine/aspartic acid+glutamic acid ratio. These may probably also affect protein digestibility and overall protein quality. They all seem to warrant further studies.