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SIMULATING PROTEIN DIGESTION ON TROUT

A RAPID AND INEXPENSIVE METHOD FOR DOCUMENTING FISH MEAL QUALITY AND SCREENING NOVEL PROTEIN SOURCES FOR USE IN AQUAFEEDS

M. Bassompierre, A. Kjær, E. McLean

Summary

A novel *in vitro* digestion system, which simulated rainbow trout gastric and intestinal digestion was developed. The method was employed to evaluate the impact of the gastric phase of digestion upon degradation of three fish meals od differing quality. Results illustrated that two–phase gastric–intestinal digestion increased the discriminatory powers of the system when compared to one–step intestinal digestion. A comparison of the system with pH–STAT methods demonstrated that the *in vitro* technique was superior. The presented method provides an ethical and cost effective means for rapid evaluation of fish meals and potentially, alternative protein sources for aquafeeds.

Key words: protein, trout, digestibility, methodology, fish meal, fish feed

INTRODUCTION

During the intensive culture of teleosts, feed costs generally represent the most important operating variable (Bassompierre et al., 1997). The single largest price component of aquafeeds is protein, generally derived from fish meal (Crampton, 1985). Fish meal quality however, may vary considerably, due to a wide range of pre–, and post–harvest factor (review: Tarr and Bieely, 1972), which affect protein digestibility. Accordingly, prior to ulti-

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mate use, the fish meal industry employs a variety of techniques to estimate protein nutritive value. In general, due to speed, cost and reproducibility, physical and chemical methods are employed. However, there is general consensus that these techniques are inadequate for accurate determination of protein nutritional value (New and Csavas, 1995). Thus, *in vivo* rat and mink digestibility trials are applied as the ultimate determinant of meal quality. However, these *in vivo* analyses are time–consuming and costly and may often be inappropriate under certain condidions (Dimes et al., 1994). It is not surprising therefore, that attempts have been made to develop *in vitro* methods for rapid evaluation of protein digestibility.

Various *in vitro* techniques have been used to examine aspects of protein digestibility in vertebrates. These system range from single–step, single–enzyme methods through to multi–phase, multi–enzyme techniques (rev iews: Boisenand Eggum 1991; Savoie 1994). However, standardisation of *in vitro* systems has proven difficult to attain for fish. The reasons for this are varied and range from lack of knowledge of fisch digestive physiology through to industrial politics. pH–STAT procedures, which employ commercially available enzymes, have been accepted by the terrestrial animal feed industry as a means of product documentation (Pedersen and Eggum, 1983). The same method has recently been examined for its use as a tool in studying fish digestive physiology (Dimes et al., 1994). However, due to its working principle the pH–STAT procedure has inherent problems which limits its industrial application. For example, high concentrations of hydrolysed product in solution can negatively affect buffering capacity and hence influence digestibility estimations (Boison and Eggum 1991). Clearly, the fish meal and aquafeed industries require quality estimators appropriate to cultured fish for in–house application and client documentation, as well as for screening novel protein sources. Development of such a system should, of course, take account of the end user of the protein. Thus, it is imperative that the development of a documentative assay should take accound of temperature, pH, digestion time, enzyme source etc. The present investigation examined the utility of a novel fish enzyme–based test, which incorporated both gastric and intestinal digestion simulations, at as a means of characterising fish meals of differing quality. The study also included chemical analyses, animal tests and pH–STAT evaluations for comparative purposes.

Table 1. Chemical, pH–STAT and rat digestibility determinations for experimental fish meals of varying soluble protein content employed by the present investigations. BV = biological value, NPU = net protein utilisation, TD = true digestibility for rat. Amino acid analyses represent g kg^{-1} meal. Titration was obtained by measuring the amount of NaOH (0.1 M) required to titrate 10 g of FM in 100 mL H_2O for 10 min. NH_3 was assessed using the Kjel–Tec–System Tecator for 10g of FM; protein, moisture, lipid and ash were quantified as described in Bassompierre *et al. (1997).*

Tablica 1. Determinante kemijske, pH–STAT, štakorske probavljivosti eksperimentalnih ribljih brašna s različitim sadržajem topljivih bjelančevina, upotrijebljene u ovim istraživanjima. BV = biološka vrijednost, NPU = neto–iskoristivost bjelančevina, TD = istinska štakorska probavljivost. Analize aminokiselina predstavljaju g kg^{-1} brašna. Titracija je određena mjerenjem količine NaOH (0.1 M) potrebne da titrira 10 g FM u 100 ml vode kroz 10 minuta. Procjena NH_3 izvršena je Kjel–Tec–system tetrakrakom za 10 g FM; bjelančevine, vlaga, masti i pepeo su kvantificirani i opisani u Bassompierre *et al. (1997.).*

Fish Meals	I	II	III
NPU	89.1	91.2	53.6
BV	90.5	92.7	54.3
TD	98.4	98.3	98.7
fat/mast	10.1	9.7	1.0
ash/pepeo	11.0	10.4	16.7
NH3	0.1	0.1	0.3
soluble protein/topljive bjelančevine	20.0	25.0	95.0
titration/titracija	67.0	53.0	146.0
protein/bjelančevine	74.4	73.6	78.8
NaCl	1.5	0.7	4.6
moisture/vlaga	7.2	7.6	7.3
pH–STAT	91.7	84.3	87.5
Gly	45.24	42.91	63.44
Cys	7.32	7.96	3.13
Met	23.22	24.70	11.88
Trp	8.51	8.85	2.50
Tyr	26.94	26.85	9.46
Lys	66.65	64.09	41.73
Asp	73.29	71.78	43.15
Val	44.08	44.71	22.70
Ile	36.95	36.97	16.54
Thr	33.67	33.50	18.89
Phe	32.19	32.57	16.50
Leu	59.16	58.06	34.72
Arg	47.66	45.60	33.63
Gln	100.72	99.08	76.40
Ser	33.75	32.25	22.33
Pro	30.86	30.01	25.55
Orn	1.08	0.58	1.26
Ala	48.18	47.62	48.1

MATERIALS AND METHODS

Enzyme extraction

Enzymes were extracted from pooled disected viscera (stomach and pyloric cecae) of farmed animals (200 g wet weight; Sydjyskdamkultur, Denmark). To 50 g of pyloric caeca was added 150 ml (3x volume) of a 10 mM phosphate 200 mM NaCl, buffer (pH 7.8; 1:3 w/v). The solution was gently stirred for 5 h (enzyme extraction) after which tissue was removed by filtering (1 mm net sieve). The resultant crude extract was then centrifuged at 15000 x *g*. Before use, and to ensure removal of low molecular weight products, the enzyme solution was dialysed overnight (cut–off 14,000 d) against 10 mM phosphate buffer, pH 7.8 and then recentrifuged at 15000 x *g*. All steps of the extraction were carried out at 5 °C. Extract chymotrypsin–like activity was assayed using the techniques described by DelMar et al. (1979) as modified by Kristjánsson and Nielson (1992). Trypsin–like activity was measured using the method described by Kristjánsson (1991). Protein in the enzyme solution was determined according to Bradford (1976) with modifications for microtiter plates (Bio–Rad 500–0001). Gastric enzymes were extracted in HCl (pH 3.8; 1:3 w/v) under gentle stirring for 3 ha at <5 °C.

Test fish meals

Three fish meals (FM) were manufactured using distinct industrial processes. FM I was obtained from a low temperature drying process, FM II was manufactured by mixing 90% press–cake with 10% spray dried soluble meal, while FM III was the soluble meal employed in the production of FM II. The chemical characteristics, rat net protein utilization (NPU) and pH–STAT digestibility prediction data accomplished according to Pedersen and Eggum (1983), are presented in Table 1.

In vitro gastric and intestinal digestion

Digestibility assessment (i. e. free amino group quantification by the trinitrobenzene sulfonic acid /TNBS/ method) was performed as described in Bassompierre et al. (1997) with the following modifications. Fifty mg of FM were stirred for 22 h in 50 ml of 50 mM acetate buffer containing chloramphenicol (1^0/000), at pH 3.8. Gastric extract (0.5 ml) was added to digestion reactors (three FM, n = 6) to perform gastric simulation. The reaction was terminated after 24 h by raising pH with the addition of 1 ml of 2.5 M NaOH. Gastric enzymes were not added to control digestion reactors (three FM, n = 6). Reactor pH was the adjusted to 7.8 with 50 ml of 250 mM phosphate buffer. After 1 hr, intestinal digestion was simulated with addition of 0.5 ml pyloric caeca extract (Suc Ala–Ala–Pro–Phe pNa activity of 2 x 2,3585 U/ml. mg protein). One set of reactors (three FM, n = 6) received

intestinal enzyme extract only. Following enzyme addition the reaction was allowed to proceed for 24 h. Controls were always incubated in an identical manner but without enzyme addition. After the reaction samples of 1 ml were collected, boiled for 5 min, rapidly cooled and stored at — 80 °C. Quantification of free amino groups was accomplished using a modification of the TNBS method. After sample centrifugation (8 min at 10000 rpm, 5 °C), 600 ml of supernatant were transferred into test tubes and incubated at 60 °C for 60 min with 2 ml of $5^0/000$ TNBS in 500 mM phosphate buffer (pH 8.2). D, L Alanine (30 mg in 50 ml buffer) was employed to construct standard curves (0–200 ml in triplicate adjusted to 600 ml). Reactions were terminated by adding 1000 mlof a 1 M HCl. Samples were measured spectrophotometrically at 420 mm.

Statistical analyses

All data relating to *in vitro* digestion were analysed by one–way ANOVA followed by pair–wise multiple comparison procedures (Student–Neuman–Keuls, Sigma Stat, Jandel Scientific).

RESULTS AND DISCUSSION

The present studies were undertaken to examine the potential value of *in vitro* protein digestion as a method of determining fish meal (FM) quality. Moreover, since most *in vitro* digestion assays employ single phase gastric (Faithfull, 1984) or intestinal (Bassompierre et al. 1997) stages, the present investigation was designed to evaluate the importance of the gastric phase during digestion. The *in vitro* digestion method used here provided amino group (AG) quantification for each of three test FM before digestion, following intestinal simulation and after gastro–intestinal digestion (Table 2). The raw material used for the production of FM III was, as indicated by the ammonia content (Table 1), low grade and contained a high level of water–soluble protein (95%, Table 1), when compared to FM I and II. This resulted in a significantly ($P<0.001$) higher level of AG in FM III before enzyme addition, when compared to the other FM (300% and 400% greater than for FM I and FM II respectively). A significant difference ($P<0.001$) in pre–digestion AG liberation was also recorded between FM I and FM II, with the former 30% greater than the latter. Following addition of intestinal enzyme extracts only, the *in vitro* system was able to separate FM I and II from FM III ($P<0.001$) however, no differences were apparent in AG liberation between FM I and FM II. This result likely occurred due to the presence of high levels of soluble protein in FM III pre–digestion.

The prec–processing of FM using gastric enzymes irrespective of FM quality, significantly increased ($P<0.05$) protein degradation, as assessed by AG liberation. One of the major roles of the stomach is considered to be the

preparation of food for further hydrolysis in the intestine (Ganapathy *et al.* 1994); with partial hydrolysis of proteins to peptides. The present results are in accord with this suggestion and corroborate the findings of Grabner and Hofer (1985), who reported an 11–27% hydrolysis of soya bean and brodad bean protein respectively, by rainbow trout pepsin 15 h post–incubation in vitro. In contrast to intestinal simulation alone, the incorporation of a gastric phase of digestion permitted individual separation ($P<0.01$) of the three FM types (Table 2), with FM II and FM III exhibiting 93% and 42% AG liberation when compared to FM I (Table 2).

In the present study, the multi–enzyme pH–STAT method ranked the three FM samples in an opposite order relative to *in vivo* rat net protein utilization (NPU) trial (Table 1), illustrating the problems of pH–based methods, which are sensitive to the buffering capacity of water soluble proteins. Similar observation have been reported by Dimes et al. (1994). A comparison of the two–step in vitro system employed herein, with that of the pH–STAT system, underlines the advantages of the presented in vitro method. Indeed the in vitro system was in better agreement with the rat NPU trials than the pH–STAT, particularly for FM I and II versus FM III. Moreover, the ranking ability of the in vitro procedure corresponded well with the presented chemical analyses (Table 1). The present investigation thus illustrates the importance of the gastric phase in simulating digestive processes in salmonid and other stomach containing species. From an applied perspective however, for example in the routine monitoring of FM quality or the evaluation of alternate protein sources, the expense and time penalty incurred with the addition of the gastric phase would likely be unwarranted, unless pruduct characterisation required more in–depth analysis. The final validation of either single or two–step digestion will nevertheless require direct comparison with in vivo trials using the end consumer.

Since the protein component of aquadiets is the single most expensive portion, a number of investigations have examined the potential of cheaper, alternative sources, both animal and plant derived (see. Kaushik, 1990; Mayer and McLean, 1995). Often, such studies have used *in vivo* trials with fish with the consequence that considerable costs are incurred. The method described by the present study may provide a rapid and economic, as well as ethical means of rapidly examining candidate protein sources. Enzyme extracts used in this study are available upon written request.

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Table 2. Gastric and intestinal digestion of three fish meals (FM) of differing quality following in vitro simulation. Protein digestion is presented as mean + SEM in amino group (AG) equivalents, quantified by the TNBS method. Data expressing the same superscript in a column were not significantly different. AG liberation = AG post-digestion value minus AG pre-digestion value.

Tabilca 2. Živčana i crijevna probava triju vrsta ribljeg brašna (FM) različite kvalitete s pomoću simulacije in vitro. Probava je bjelančevina prikazana kao srednja vrijednost +SEM u ekvivalentima amino-grupe (AG), kvantificirana putem TNBS metode. Podaci označeni istim slovom u svakom redu nisu signifikantno različiti. AG oslobađanje = AG vrijednost nakon probave minus AG vrijednost prije probave.

	Undigested control: Ag pre-digestion / Neprobavljena kontrola: AG prije probave	AG post-intestinaldigestion / AG nakon crijevne probave	AG post-gastro-intestinal digestion / AG nakon želučano-crijevne probave	Intestinal AG liberation / Crijevno AG oslobađanje	Gastro-intestinal AG liberation / Želučano-crijevno AG oslobađanje
FM I	$0.626^{a} \pm 0.03$	$3.33^{a} \pm 0.08$	$4.35^{a} \pm 0.05$	$2.70^{a} \pm 0.08$	$3.72^{a} \pm 0.05$
FM II	$0.459^{b} \pm 0.02$	$3.04^{a} \pm 0.08$	$3.95^{b} \pm 0.07$	$2.58^{a} \pm 0.08$	$3.49^{b} \pm 0.07$
FM III	$1.853^{c} \pm 0.04$	$3.08^{a} \pm 0.08$	$3.42^{a} \pm 0.05$	$1.23^{b} \pm 0.08$	$1.56^{c} \pm 0.05$

Sažetak

SIMULACIJA PROBAVE BJELANČEVINA PASTRVA

BRZA I JEFTINA METODA ZA ODREĐIVANJE KAKVOĆE RIBLJEG BRAŠNA I PRIKAZIVANJE NEISPITANIH IZVORA BJELANČEVINA U HRANI ZA VODENE ORGANIZME

Ovaj rad prikazuje novi *in vitro* probavni sustav, koji simulira želučanu i crijevnu probavu u pastrva. Metoda je razvijena kako bi vrednovala utjecaj želučane faze probave na degradaciju triju vrsta ribljega brašna različite kakvoće. Rezultati pokazuju da dvofazna gastrointestinalna probava povećava diskriminatornu snagu sustava u usporedbi s jednofaznom crijevnom probavom. Tehnika *in vitro* pokazala se superiornom i u usporedbi sa sustavom pH–STAT. Prikazana metoda osigurava etički i finacijski pogodno sredstvo za brzo vrednovanje ribljega brašna, a potencijalno i drugih izvora bjelančevina u hrani za vodene organizme.

Ključne riječi: bjelančevine, pastrva, probavljivost, metodologija, riblje brašno, riblja hrana

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