Effect of 1-Propanol on the Activity of Intestinal Proteolytic Enzymes of the European Sea Bass *Dicentrarchus labrax*

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The effect of 1-propanol and aliphatic alcohols on intestinal proteolytic and peptidase activities of the European sea bass *Dicentrarchus labrax* was examined in vitro. Results show that 1-propanol has a definite enhancing effect on the activity of trypsin and carboxypeptidase B as well as on the total proteolytic activity when tested with casein. Other intestinal enzymes were inhibited to various extents by 1-propanol. Effects of other aliphatic alcohols (e.g., butanols, 2-propanol, ethanol, and methanol) were tested as well. Results show that the less polar alcohols brought about the highest amplification of trypsin activity.

INTRODUCTION

Aliphatic (simple) alcohols have been shown to enhance the activity of different enzymes in plant as well as animal tissues (Siegel et al., 1961; Shoseyov et al., 1988; Honjo et al., 1990). In contrast, compound alcohols did not show such enhancing effects (Siegel et al., 1961). Fish require large quantities of protein in their diet; typical artificial diets for predatory fish will contain up to 60% protein (Hepher, 1988). Therefore, enhancement of the proteolysis efficiency in fish intestines is of importance for optimal rearing. Only a limited number of investigations have addressed the possibility of utilizing various agents to enhance the proteolytic activity existing in the guts of aquatic organisms by inducing a better solvent medium wherein these reactions occur (Honjo et al., 1990).

The exact nature of the interactions among the enzymes, substrate, and hydrophobic surrounding induced by the addition of various alcohols has not been thoroughly investigated.

In the present study the effects of various aliphatic alcohols on fish intestinal enzymes and their specific and nonspecific substrates were evaluated. This was done to explore the possibility of utilizing them or other aliphatic compounds to enhance fish intestinal protease and peptidase activities. Special emphasis was given to the use of 1-propanol since this simple alcohol, which is more hydrophobic than methanol or ethanol, is still fairly miscible with water and its effect can be investigated over a wide range of concentrations. In addition, the LD_{50} of 1-propanol, when verified using rats by oral administration, was found to be 1.87 g/kg, while that of 2-propanol was 5.8 g/kg (Strecher, 1968). These levels do not exclude the investigation of future use of this alcohol in fish feeds.

MATERIALS AND METHODS

Fish. The marine euryhaline fish *Dicentrarchus labrax* was reared for several months in our laboratory, in freshwater, under controlled conditions at 23 ± 2 °C. Fish were fed twice a day ad libitum on an artificial diet containing 40% protein. The last feeding took place 4 h prior to sacrifice and extraction of intestines to obtain maximum secretion of enzymes in the lumen.

Preparation of Homogenates. Intestines were removed from three to four adult fish weighing about 200 g each. All preparation work was carried out at ~4 °C. The cooled intestines were blended at low speed in a Waring blender with 20 volumes of 50 mM Tris-HCl buffer containing 20 mM $CaCl_2$ and 50 mM KCl, at pH 8.0, by applying several short blending periods of 15 s each. The homogenate was then centrifuged at 25000*g* for 30 min, and the supernatant was filtered through glass wool.

Statistical Analysis. Data were subjected to one-way ANOVA. Whenever *F*-test first showed a significant difference in means, a *t*-test procedure was made to compare means pairwise.

Enzymatic and Chemical Assays. Unless otherwise stated, all chemicals and artificial substrates were obtained from Sigma Chemical Co. (St. Louis, MO). Hammarsten's casein was obtained from BDH Biochemicals. The experiments were replicated at least three times using independent homogenates. All enzymatic assays were conducted in triplicate. Enzymatic activities were measured at 30 °C. Trypsin-like and chymotrypsin-like activities were measured using the substrates benzoyl-DL-arginine *p*-nitroanilide (DL-BAPNA) according to the method of Fritz et al. (1974) and glutaryl-L-phenylalanine *p*-nitroanilide (GPNA) according to the procedure of Erlanger et al. (1966), respectively. Carboxypeptidase A was measured using *N*-(2-furanacryloyl)-L-phenylalanyl-L-phenylalanine (FAPP) as substrate according to the method of Riordan and Holmquist (1984), and carboxypeptidase B was measured with furylacryloyl-L-alanyl-L-lysine (FAAL) as substrate according to the procedure of Skidgel and Erdos (1984). Elastase was measured according to the procedure of Feinstein et al. (1973), utilizing *N*-acetyl-(alanine)$_3$ *p*-nitroanilide ($NAcA_3NA$) as substrate. Leucine aminopeptidase was measured at pH 8.0 with leucine *p*-nitroanilide (LNA) as substrate, similarly to the measurement of trypsin-like activity with BAPNA. Collagenase activity was measured with the *N*-2,4-dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg (DNP-peptide) as substrate according to the method of Masui et al. (1977). The results were expressed as specific activity: nanomoles of substrate split per minute per milligram of protein.

Caseinolytic activity was measured at 35 °C on 0.6% casein (Hammarsten) dissolved in borate buffer according to the procedure of Rick (1974). To 1.9 mL of casein solution at pH 8.0 containing 5 mM of $CaCl_2$ was added 0.1 mL of homogenate. Following incubation for 10 min, the reaction was terminated by the addition of 3 mL of trichloroacetic acid (TCA). Following a holding period of at least 1 h, the tubes containing the inactivated reaction mixture were centrifuged for 15 min at 1100*g*. The material in the supernatant which was reactive against Folin reagent was measured by the method of Lowry et al. (1951) with tyrosine as standard. The results are expressed as nanomoles of tyrosine per minute per milligram of protein.

To measure the effect of added alcohols, portions of the homogenate were preincubated for 30 min at the indicated alcohol concentration and the alcohol was also included in the reaction mixture. Inhibition by the trypsin inhibitor, tosyllysine chloromethyl ketone (TLCK), was measured on homogenate portions incubated for 30 min with or without 1-propanol at room temperature, prior to the addition of the inhibitor (1 mM). The

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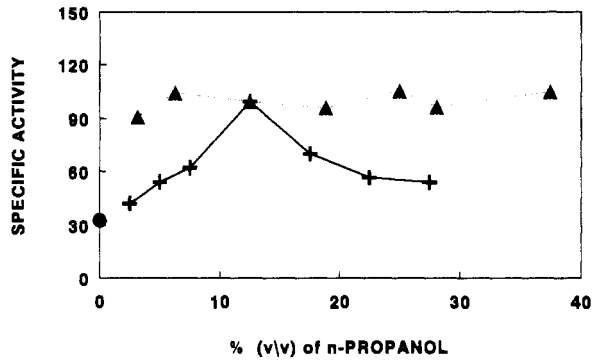

Figure 1. Effect of 1-propanol at various concentrations in preincubation and reaction media on trypsin-like activity of *D. labrax* intestinal homogenate: (▲) various concentrations in preincubation medium and 12.5% v/v in reaction medium; (+) 12.5% v/v in preincubation medium and various concent rations in reaction medium; (●) no 1-propanol added to reaction and preincubation media. Specific activity expressed as nanomoles of BAPNA per minute per milligram of protein.

reaction rate was measured after an additional incubation of 30 min at room temperature. Inhibition by soy trypsin inhibitor (STI) was measured after preincubation of the homogenate for 30 min with 1 mg/mL inhibitor.

Purification of Trypsin from the Crude Homogenate. Ammonium sulfate was added to the crude homogenate so as to obtain 60% saturation. The precipitate was dissolved in 25 mM Tris-HCl buffer at pH 8.0 and dialyzed against distilled water for 18 h. The retained solution was centrifuged at 20000*g* and lyophilized. The lyophilized material was dissolved in Tris-HCl buffer containing 25 mM KCl and 10 mM $CaCl_2$ and further purified by affinity chromatography on a prepacked *p*-aminobenzamidine (PABA)–agarose column (Sigma PAB-5) of 2.5 mL. The column was loaded with 0.9 mL of solution containing 30 mg of protein. Trypsin was eluted from the column by 100 mM acetic acid at a flow rate of 9 mL/h. Fractions of 3.0 mL were collected into tubes containing 0.45 mL of 1 M Tris-HCl buffer and 10 mM $CaCl_2$. The fractions containing tryptic activity, measured with BAPNA as substrate, were combined and used for measurements of the effect of alcohol on the proteolytic and BAPNA splitting activity. Purity of this preparation was also checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). This was carried out in 12.5% acrylamide gels according to the method of Laemmli (1970).

Assay of Protein. Protein was assayed according to the method of Lowry et al. (1951) with bovine serum albumin (BSA) as standard.

RESULTS

The effect of 1-propanol on the trypsin-like BAPNA splitting activity of crude intestinal homogenates is shown in Figure 1. Maximum amplification of 205% (3-fold increase) was obtained at concentration of 12.5% v/v of propanol in the reaction mixture. From the results presented in Figure 1 it is evident that preincubation of the homogenate with a wide range of propanol concentrations (0–40% v/v) has no effect on the BAPNA splitting activity. The amplification of the activity depends only on the concentration in the reaction mixture. This suggests that the decrease in activity at concentrations exceeding 12.5% v/v in the reaction mixture is not due to irreversible inactivation of the trypsin-like enzyme.

No effect of propanol on the absorption coefficient of *p*-nitroaniline, the chromophore released by the reaction with *p*-nitroanilide substrates, was observed. Changes in K_m (Michaelis–Menten constant) and in V_{max} of this reaction were inspected using a Lineweaver–Burk curve, following passage from 0% to 12.5% v/v of 1-propanol in the reaction mixture. A major increase in V_{max}, from 639 to 1307 nmol of substrate min^{-1} (mL of intestinal homogenate)$^{-1}$ was observed. In addition, K_m was increased from 0.201 to 0.482 mM when 12.5% 1-propanol was present in the reaction mixture.

The effects of 1-propanol and 1-butanol on various enzymatic activities of crude homogenates on synthetic substrates are shown in Table 1. These two alcohols were found to enhance the trypsin-like BAPNA splitting activity and to a lesser extent the carboxypeptidase B activity on FAAL. 1-Propanol and 1-butanol at concentrations of 12.5% and 6.2% v/v, respectively, caused strong inhibition of collagenase, elastase, and leucine aminopeptidase activities. The concentration of propanol used in the experiments presented in Table 1 was chosen as the concentration yielding maximum amplification of the homogenate activity on BAPNA (Figure 1). The concentrations of 1-butanol and *n*-amyl alcohol tested were dictated by their limited solubility in water. In the case of *n*-amyl alcohol, which is only slightly miscible with water, significant amplifications of trypsin-like and carboxypeptidase B activities were still observed but the inhibiting effect on other activities is less pronounced. The BAPNA splitting activity of intestinal homogenates derived from *D. labrax* was totally annihilated by both STI and TLCK, thus confirming that this activity is indeed tryptic activity. Using 12.5% v/v 1-propanol in the preincubation (prior to incubation with inhibitor) and reaction media did not protect the tryptic activity of the homogenate from inhibition by STI and TLCK. Only weak chymotrypsin-like activity was detected in the homogenates in the presence or absence of 1-propanol.

To verify that interaction between trypsin and the alcohols is involved in their amplification of the activity on BAPNA, the activity of trypsin, purified from intestinal homogenates by affinity chromatography, was measured in the presence of 12.5% 1-propanol. The purified trypsin migrated as a single band of MW of 26 400 on SDS–PAGE. Amplification of 118% of this purified fraction's activity on BAPNA was obtained in the presence of 12.5% 1-propanol. Specific activity without 1-propanol was 1295

Table 1. Effect of Alcohols on Enzymatic Activities of Intestinal Homogenate of *D. labrax*[a]

		alcohol tested							
enzyme	substrate	homogenate, no alcohol	SD[b]	1-propanol, 12.5% v/v	SD	1-butanol, 6.2% v/v	SD	*n*-amyl alcohol, 1.8% v/v	SD
trypsin	BAPNA	44.7	6.5	93.2	14.7	90.7	11.3	73.2	5.9
chymotrypsin	GPNA	0.8	0.2	0.5	0.3	0.7	0.4	0.6	0.4
elastase	$NAcA_3NA$	17.9	1.2	4.1	0.3	4.3	1.2	14.1	1.1
collagenase	DNP-peptide	16.2	3.0	2.8	0.6	3.1	1.1	7.7	1.2
carboxypeptidase A	FAPP	33.6	4.1	13.1	4.8	16.4	6.3	23.9	6.0
carboxypeptidase B	FAAL	10.1	1.9	15.1	2.3	14.1	1.1	15.4	2.2
leucine aminopeptidase	LNA	13.2	2.3	1.7	0.1	1.1	0.5	12.4	1.4

[a] Specific activities: nanomoles of substrate per minute per milligram of protein. [b] SD, standard deviation (based on data obtained from three independent experiments, each was conducted in triplicate).

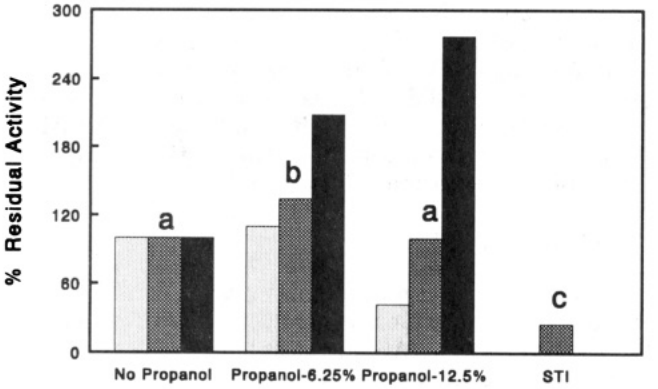

Figure 2. Effect of 1-propanol and STI on caseinolytic and BAPNA splitting activities of crude homogenate and purified fish trypsin: (lightly shaded bar) purified trypsin on casein; (heavily shaded bar) crude homogenate on casein; (black bar) purified trypsin on BAPNA. STI was tested on casein only with the crude homogenate. Bars with the same letter heading are not significantly different at $P < 0.05$ level. All others are significantly different at $P < 0.01$.

Table 2. Amplification of Fish Intestinal Trypsin Activity by Various Alcohols[a]

alcohol	molarity	% v/v	rel % activity
b			100[c,d]
glycerol	0.75	5.46	91[d]
propylene glycol	0.75	5.46	92[d]
methanol	0.75	2.99	110[d]
ethanol	0.75	4.30	125
2-propanol	0.75	5.61	142
1-propanol	0.75	5.61	185
2-methyl-2-propanol	0.75	6.92	205
2-butanol	0.75	6.92	260
1-butanol	0.75	6.92	300

[a] Activity measured on BAPNA as substrate. [b] No alcohol added to homogenate. [c] Specific activity: 400 nmol of BAPNA min^{-1} (mg of protein)$^{-1}$. [d] Not significantly different at $P = 0.01$.

nmol of BAPNA min^{-1} (mg of protein)$^{-1}$, while in the presence of 12.5% 1-propanol it was 2826 nmol of BAPNA min^{-1} (mg of protein)$^{-1}$.

The caseinolytic activity of intestinal homogenates is strongly inhibited by STI, suggesting that this activity is mainly due to trypsin. To fathom the interactions among the trypsin-like enzyme of the fish, the casein substrate, and the surrounding hydrophobicity, the activity of purified trypsin-like fish enzyme was evaluated on casein and BAPNA in the presence of various 1-propanol percentages. Results show that the optimal 1-propanol concentration for the caseinolytic activity (~6%) is below that observed for BAPNA activity (~12%) but is very similar to that observed when the crude homogenate was used (see Figure 2). In addition, the caseinolytic activity of the purified enzyme was amplified to a smaller extent (~10%) than the activity of the crude homogenate (~35%). This was in spite of the fact that the purified enzyme fraction yielded a higher amidolytic activity on BAPNA [360 nmol of substrate min^{-1} (mL of sample)$^{-1}$] than the crude homogenate [100–145 nmol min^{-1} (mL of sample)$^{-1}$]. The optimal 1-propanol concentration for the caseinolytic activity is thus below the optimal concentration for BAPNA activity (Table 1 and Figure 2).

The amplification of the BAPNA splitting activity by various alcohols is presented in Table 2. Comparison of the activity amplifications at similar molar or volume concentrations of alcohols shows that the less polar substances amplify this activity more than the polar ones. Thus, at concentration of 0.75 M, 1-butanol amplifies more than the C_4 alcohols which are more polar, while methanol causes no amplification. Similarly, the less polar 1-propanol amplifies the activity on BAPNA more than 2-propanol. In accordance with this trend, neither glycerol nor propylene glycol yielded amplification of the activity.

Table 3. Effect of 5% (v/v) 1-Propanol on Trypsin Originating from Different Animals

enzyme origin	specific activity[b] no propanol added	specific activity[b] 5% propanol added	% amplification by propanol
fish *D. labrax*[a]	98	146	49
bovine	1790	2166	21
hog	3718	3920	5

[a] Fish trypsin activity was derived from crude intestinal homogenate. [b] Specific activity was defined as nanomoles of nitroaniline released per minute per milligram of protein. Differences in the specific activities as well as in amplification levels induced by propanol are significant ($P < 0.01$).

The effects of 5% 1-propanol in the reaction mixture on trypsin from crude intestinal fish (*D. labrax*) homogenate and on purified hog and bovine trypsins were compared (Table 3). Whie hog trypsin remained almost unaffected, bovine trypsin and trypsin purified from fish homogenate showed amplifications of 21% and 49%, respectively, of the activity on BAPNA.

DISCUSSION

The results presented in this work suggest that aliphatic alcohols stimulate mainly the tryptic activity of fish intestinal homogenates, measured as its amidolytic activity on BAPNA or as proteolytic activity on casein. Other enzymatic activities are either stimulated to a smaller degree, namely carboxypeptidase B, or inhibited to various extents (see Table 1). The effect of 1-propanol varies depending on the origin of the tested trypsin. Of the three trypsins tested, that of *D. labrax* showed the highest amplification in the presence of 1-propanol (Table 3). However, 1-propanol did not protect the trypsin activity from being inhibited by typical trypsin inhibitors such as STI and TLCK.

The results obtained with trypsin purified from the fish intestinal homogenates suggest that the alcohols directly affect the reactivity of the enzyme and do not act indirectly by removing a disturbance to the enzymatic reaction present in the crude homogenate.

The amplification of the activity seems to be connected with hydrophobicity of the alcohols. Thus, 1-butanol and 1-propanol, which are the less polar in their respective families of alcohols, have a more pronounced stimulatory effect on the trypsin-like activity than the more polar alcohols. No effect was observed with methanol at concentrations at which stimulation was observed with higher alcohols.

By reaching a concentration of 12.5% 1-propanol in the reaction media, using BAPNA as substrate, a maximal amplification of amidolytic activity level is achieved. On the other hand, when casein was used as a substrate, the maximal amplification of proteolytic activity was reached at 6.25% (see Figure 2). The fact that different amplification levels are obtained for BAPNA splitting and caseinolytic activities is not surprising since we are dealing with two different hydrolytic reactions, one splitting an amide bond in a low molecular weight substance and the other splitting a peptide bond in a high molecular weight protein. These two reaction types seem to respond differently to changes in the hydrophobicity of the medium.

A change of the enzyme conformation in the more hydrophobic medium has been suggested as the reason for the change in tryptic activity in crustacean guts observed in the presence of 1-propanol by Honjo et al. (1990). These results are confirmed in our work; i.e., with enhanced hydrophobicity of the reaction medium, by using different aliphatic alcohols, improvement of amidolytic and caseinolytic activity was achieved (Table 2; Figure 2). In the case of caseinolytic activity, changes in the substrate conformation may occur as well. It is suggested that when a level of above 6.25% 1-propanol exists in the reaction mixture, the conformation changes in the casein structure cause inhibition to the caseinolytic activity. In addition, we might speculate that the casein itself contributes to the hydrophobicity more than the synthetic substrate, due to its amino acid composition and fat content.

It is evident that the contribution of trypsin to the proteolytic process in the fish intestine is of major importance, constituting about 75% of the total activity (see Figure 2, effect of STI treatment). However, the amplification of caseinolytic activity observed with crude homogenate is probably due not only to the effect on trypsin but also to the effect on the whole enzymatic cascade which operates on the products of trypsin activity. Carboxypeptidase B activity is also amplified (Table 1), while other enzymes might be inhibited only to a minor extent at a level of 6.25% 1-propanol. This might explain the 10% vs 34% amplification, in the presence of 6.25% 1-propanol, of purified *D. labrax* trypsin and of the crude homogenate consequently. This hypothesis is supported by the findings which show that purified fish trypsin had amidolytic activity of 360 nmol of split substrate min^{-1} $(mL\ of\ sample)^{-1}$, while the amidolytic activity of the crude homogenate was in the range 100–145 nmol of split substrate min^{-1} $(mL\ of\ sample)^{-1}$. Hence, it seems that the composition of the enzymatic orchestra is important to obtain better amplification.

1-Propanol was utilized in this work as a substrate demonstrating the effect of enhanced hydrophobicity in reactions of proteolytic activity. Although we are aware of the limitations that exist in the addition of such an ingredient in fish feeds, it should not be totally excluded. Some publications summarizing work conducted with other vertebrates show that the effects of 1-propanol administered by inhalation are not harmful at moderate levels (Nelson et al., 1989). However, fish-feeding experiments still require methodologies to be developed to prevent absorption of the tested alcohols or hydrophobicity-inducing agents prior to their arrival at the intestinal section of the digestive tract.

Trypsin constitutes the main intestinal proteolytic activity in *D. labrax*; therefore, the possibility of increasing its activity by various feed additives is important. The protein digestive capacity of the intestinal tracts is the result of various proteolytic and peptidase activities present in them. Since several activities were found to be inhibited under conditions that stimulated tryptic activity, the overall nutritional implications of the results reported here are not self-evident and must be further investigated. It should therefore be interesting to test the hypothesis that different fish feeds will induce different hydrophobicity in the lumen due to their amino acid composition and fat ingredients, thus differentially affecting the proteolytic process.

ABBREVIATIONS USED

DNP-peptide, *N*-2,4-dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg; DL-BAPNA, benzoyl-DL-arginine *p*-nitroanilide; GPNA, glutaryl-L-phenylalanine *p*-nitroanilide; FAAL, furylacryloyl-L-alanyl-L-lysine; FAPP, *N*-(2-furanacryloyl)-L-phenylalanyl-L-phenylalanine; LNA, leucine *p*-nitroanilide; $NAcA_3NA$, *N*-acetyl(alanine)$_3$ *p*-nitroanilide; STI, soy trypsin inhibitor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLCK, tosyllysine chloromethyl ketone.

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