It was thus found that the maximal degree of protein hydrolysis would be reached at the following settings of the enzymatic reaction: temperature 50°C, duration 1 to 3 hours, hydromodulus 1:1.

Absorption of protein hydrolysis products from the low-value freshwater fishes in question (smelt, ruff, bleak) was maximized at 300 nm, whereas earlier results (Mukhin and Novikov, 2001) of selection of optimal conditions for proteolysis in marine invertebrates suggested absorption of protein hydrolysis products peaked at 280 nm. Marine invertebrates are known to have a hypertonic environment (osmotic pressure in their tissues is much lower than the osmotic pressure of sea water) and the range of higher temperatures than our study objects – low-value freshwater fishes, which, on the contrary, feature a hypotonic environment and the range of lower temperatures.

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PROTEINASE COMPLEX FROM FRESHWATER FISHES OF POTENTIAL RESOURCE VALUE IN KARELIA

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People have been aware of nutritional and pharmacologic properties of aquatic organisms since olden times. The uses of biologically active macromolecules derived from aquatic, mainly marine, organisms in human and veterinary medicine, microbiology and various industries, especially in the food and forage industries, are multiple. Freshwater organisms have been less studied in this sense. At the same time, the fish fauna of lakes in Republic of Karelia comprises quite a number of so-called low-value species ("of potential resource value") such as ruff, perch, smelt, stickleback, bleak, which can be viewed as raw material for various biologically active substances, including proteolytic enzymes. Let us stress that available published data on extraction and utilization of proteolytic enzymes from aquatic organisms are mainly concerned with marine organisms (Kuchina et al., 2007; Mukhin and Novikov, 2001; Mukhin, 2003; Stein et al., 2005).

In view of the above, we undertook to separate, purify and describe the proteinase complex from some low-value fishes (smelt – *Osmerus eperlanus eperlanus* (L.), ruff – *Acerina cernia* (L.), bleak – *Alburnus alburnus* (L.) inhabiting waters of Karelia.

Tissues of the organisms in question were homogenized in the 1:2 ratio in the chosen extraction medium (0.1 M KCI) in a MPW-324 homogenizer. After the homogenate had been refrigerated for 3 hours, filtered and centrifuged twice (10,000 g x 30 min, K-24; 30,000 g x 60 min, Optima LE – 80 K), the activity of proteolytic enzymes was determined in the supernatant fluid in the pH range of 2.5 to 8.5. The activity of the enzymes was expressed in units of optical density of the solutions containing the products of hydrolysis of various substrates at 37°C for 1 hour per 1 g of wet weight. The optical density of the solutions was measured spectrophotometrically at 280 nm (protein substrate hydrolysis); at 410 nm (specific elastase substrate hydrolysis); at 525 nm (specific cathepsin B substrate hydrolysis). Proteinase activity was determined following a modified Anson's technique (Alekseenko, 1968): for cathepsin D – through hydrolysis of 1% hemoglobin solution at pH = 3.6; for cathepsin E – through hydrolysis of 1% bovine serum albumin solution at pH = 2.5; for neutral and alkali proteinases – through hydrolysis of 1% casein solution at pH = 7.0 and 8.5, respectively. Cathepsin B activity was determined by decomposition of

the ethyl ester N – benzoyl – DL – arginine, pH 5.0 (Barrett and Heath, 1977). Elastase activity was determined after Feinstein (1973), with Suc – Ala – Ala – Ala – p – NA as the substrate.

Protein concentration in the samples was determined spectrophotometrically using Bradford (1976) assay. We used the resultant data in the process of separation and partial purification of proteinases to estimate specific activity of enzymes, which was referenced to 1 mg of protein.

Gel chromatography. All assaying was done in the cold room (+4°C). Sephadex G-100 and standard equipment by Pharmacia and LKB (Sweden) were used in chromatography. The columns were prepared and packed with Sephadex by the modified Flodin's method, recommended by Pharmacia. Sephadex G-100 swelling and rinsing were performed as described in the manual by Determan (1970).

The resultant data were processed by conventional variation statistics methods (Ivanter and Korosov, 2003). The differences were compared through Pearson's chi-square test using Statgrafics 2.0 software for Windows (Korosov and Gorbach, 2007).

As the result, proteinase activity maxima in all study objects were detected both in the acidic (pH 2.5–5.0) and in the weakly alkaline (pH 7.2–8.0) regions (Fig. 1). Owing to the presence in the cell of two types of hydrolases, active in the neutral and the acidic pH regions, the organism is provided with a wider variety of intracellular enzymes (Nemova, 1991). A known fact is that proteinases which pH optimum is in the acidic region would be found lysosome-like structures, whereas neutral proteinases are located in the intracellular fluid (Mosolov, 1971).

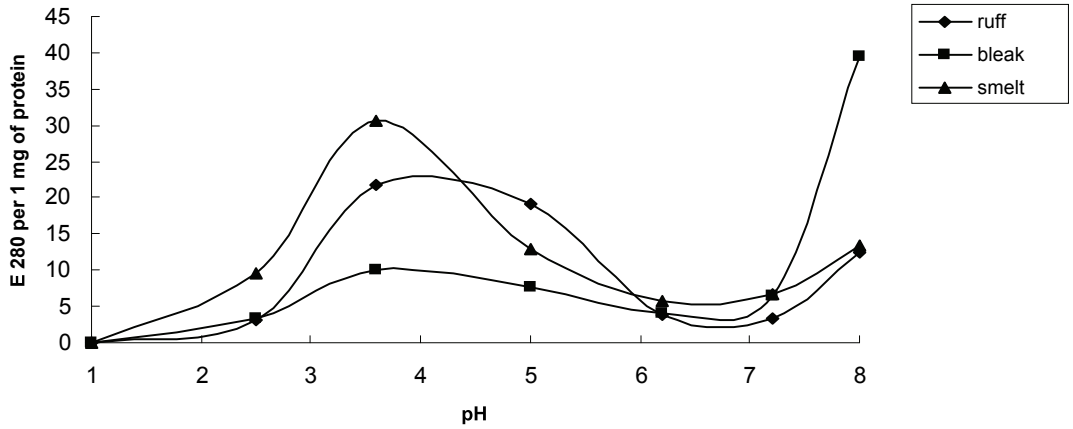

Fig. 1. Total proteolytic activity in minced low-value fishes at different ambient pH values

One can see from Fig. 1 that the proteolytic activity maximum in ruff and smelt homogenates was higher at acid pH values than at a high pH value. The situation with bleak is the opposite: the contribution of alkali proteinases to total activity is greater than that of acid proteinases. These distinctions may be due to eco-physiological characteristics of the fish species. Thus, bleak, in contrast to ruff and smelt, mainly stays in upper water layers, spawns quite late (June-July), and has different feeding habits (does not predate like ruff or smelt). The sample processing procedure might have influenced also: unlike ruff and smelt, which were assayed almost immediately after capture, bleak was stored frozen for a while, and the process of autolysis might have begun in the material. We plan to continue this work and choose certain comparable conditions for storage of the study material.

The graphs in Fig. 2 demonstrate the activity of proteolytic enzymes in ruff homogenate at an ambient pH of 2.5 to 7.7. The proteolytic activity maximum at pH 3.6 (hemoglobin as the substrate) appears to be connected with the lysosomal proteinase cathepsin D (Pohl et al., 1981; Press et al., 1960). The differences are significant (Pearson's chi-square test). The results of gel chromatography, where albumin, casein and immunoglobulin G were used as the markers, indicate the molecular weight of the enzyme protein in this fraction was about 40 kDa. Similar data were obtained for bleak and smelt homogenates.

The fractions with maximal hemoglobin hydrolyzing activity at pH 3.6 were then collected, concentrated in cells with UM-10 membrane (Amicon, USA) and used as the enzyme preparation which properties were further investigated.

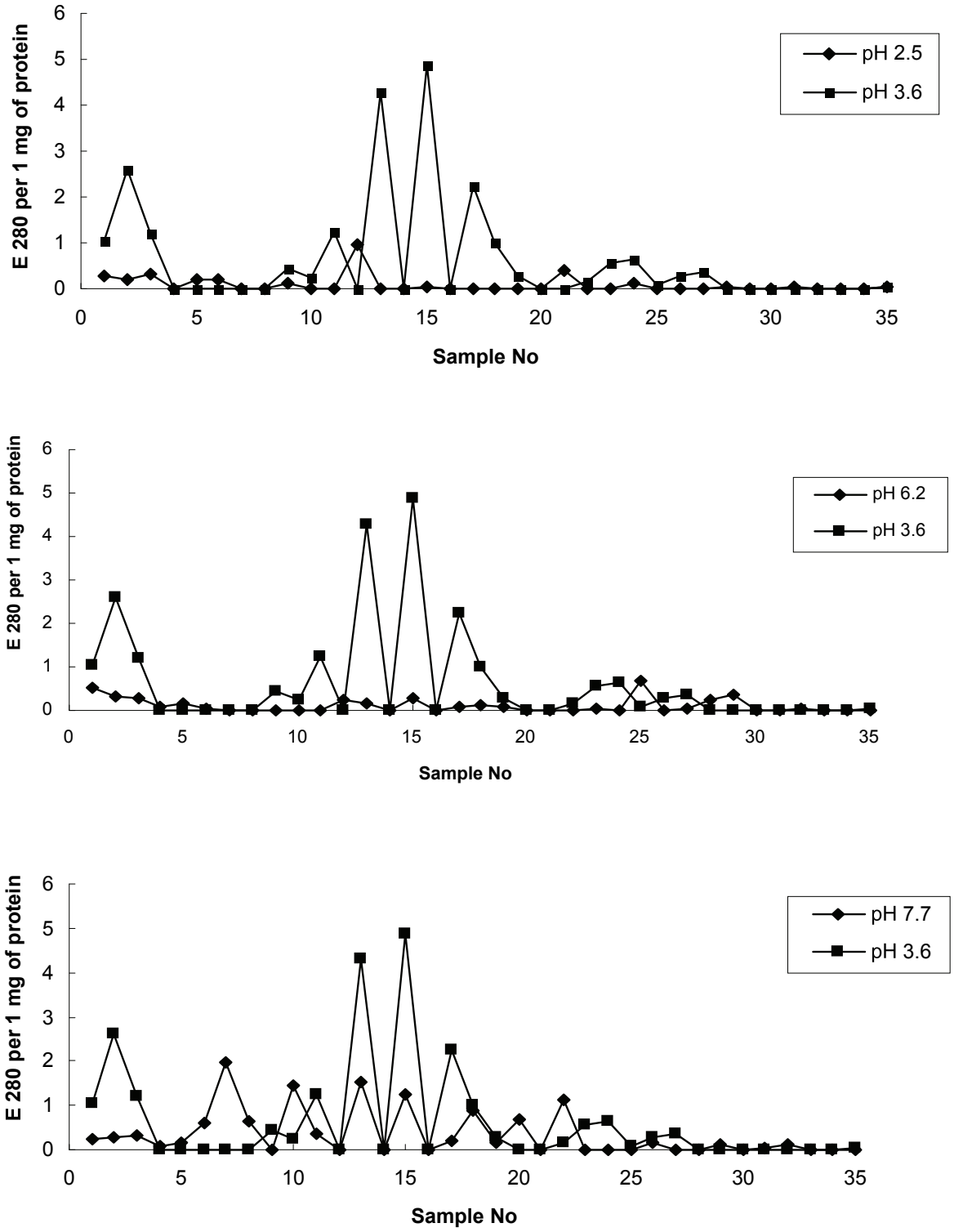

Fig. 2. Activity of proteolytic enzymes in ruff homogenate at pH 2.5–7.7

The results of application of proteinase-specific inhibitors (iodoacetate, Na_2 EDTA, Zn_2SO_4, parachloromercury benzoate (PCMB), phenylmethylsulfonyl fluoride (PMSF), Hg_2SO_4, pepstatin) indicate the range of proteolytic enzymes from the minced tissues of the fish species studied is represented at pH 2.5–3.6 by aspartyl proteinases (100% inhibition of activity by the inhibitor specific to active-site carboxyl groups – pepstatin), metalloproteinases (55–100% inhibition of activity by the inhibitors of metal ions – Na_2 EDTA and Zn_2SO_4), thiol-dependent (100% inhibition of activity by the inhibitors specific to active-

site thiol groups – mercury, iodoacetate and PCMB) and serine (100% inhibition of activity by the inhibitor specific to active-site serine groups – PMSF) proteinases (Mosolov, 1971; Barrett, 1980; Barrett and Heath, 1977).

It was found that at pH 5.0 pepstatin, iodoacetate and mercury sulphate activate the enzyme preparation (Table).

Table. Effect of various inhibitors on the activity of proteinases from smelt and bleak homogenates (% of the control)

pH	Control	iodoacetate	Na_2 EDTA	Zn_2SO_4	PCMB	PMSF	Hg_2SO_4	pepstatin
smelt								
2.5	1.99	–76	–100	–81	–87	–100	–100	–100
3.6	12.64	–80	–45	–20	–55	–31	–65	–95
5.0	6.69	50	–8	25	13	29	105	38
bleak								
2.5	0.98	–100	–100	–59	–98	–100	–100	–100
3.6	3.29	0	–33	–17	–43	–34	–80	–90
5.0	3.91	21	–63	–52	–30	–32	35	97

Thus, the characteristics of inhibition indicate the enzyme preparation produced within the study is a complex of aspartyl, serine, thiol proteinases and metalloproteinases.

Let us note in conclusion that so-called low-value fish species from lakes of Karelia can be viewed as potential raw material for composite preparations with high proteolytic activity, which can be utilized as bioactive additives to high-quality forage in fish and animal farming.

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