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Lipolytic activity of Antarctic krill, *Euphausia superba* Dana

ABSTRACT: Lipolytic activity was assayed in samples of Antarctic krill frozen in different conditions and in its liquid digesta with synthetic (tributylglycerol, esters of 2-naphtol and fatty acids C_3, C_8, C_{14} and C_{18}) and natural (olive oil) substrates. It was testified that the lipolytic activity is several-fold higher in the crustaceans with high food intake than in those with an empty digestive tract. Krill lipases show higher activity against esters of unsaturated fatty acids that against analogous derivatives of saturated ones and 10-fold higher affinity tributylglycerol ($K_m = 1.12$ mM). Their maximal activity is at pH 6.4 and 37°C. *E. superba* lipases preserve total activity up to 35°C for 45 minutes, and are completely inactivated at 55°C for 5 minutes. Prevailing part of lipolytic activity is present in krill cephalothorax, however, extracts from krill abdomen also display a marked activity. Krill lipases are probably resistant to an attack of crustacean's proteinases.

K e y w o r d s: Antarctic krill, digestive enzymes, lipases.

Introduction

Antarctic krill, *Euphausia superba* Dana is the main representative of Southern Ocean zooplankton and plays the key role in Antarctic ecosystem (Rakusa-Suszczewski 1979, Laws 1985, El-Sayed 1988, Ross and Quentin 1988). The world catching of the crustacean which is mainly converted in countries of Far East to a range of food and fodder products (Martin 1979, Murakami and Miyake 1988), as well as provides considerable amounts of chitin (Gooday 1990), does not exceed 1 mln tons per year, though the annual production of *E. superba* biomass is high and estimated to amount from 100 to 500 mln tons (Ross and Quentin 1988).

Krill's marked instability when caught, which impedes the animal's processing, prompted research into the crustacean's digestive enzymes in the late

1970's. Majority of works concern peptide hydrolases of *E. superba*, amongst which the most active and heterogenous are serine proteinases (EC 3.4.21) (Chen, Yang and Chen 1978, Kimoto Kusama and Murakami 1983, Kimoto, Yokai and Murakami 1985, Osnes and Mohr 1985, Bucht and Karlstam 1986, Karsltam, Johansson and Bryno 1991, Turkiewicz, Galas and Kalinowska 1991). It was testified that they play the main role in a process of crustacean's autolysis *post mortem* (Kawamura *et al.* 1984, Kołakowski 1986, Ellingsen and Mohr 1987).

Results of studies on krill digestive enzymes enabled to invent suitable methods of *E. superba* storage and processing, as well as confirmed an omnivoreity of the crustacean suggested by some biologists (Boyd, Heyraud and Boyd 1984, Ikeda 1984, Hopkins and Torres 1989). Till 1980's Antarctic krill was considered to be one of few phytophages in Euphausiacea (Mauchline and Fisher 1969, Ligowski 1982). Some enzymes, i.e. a collagenolytic serine proteinase detected in *E. superba* by Turkiewicz, Galas and Kalinowska (1991) the presence of which points to the possibility of animal proteins digestion by krill, and well developed system of digestive glycosidases with prevailing activities of two endo-β-1,3-glucanases (EC 3.2.1.39), described by Turkiewicz, Galas and Zielińska (1985), and Suzuki *et al.* (1992) might be considered to be enzymes-markers of Antarctic krill omnivereity. The latter two enzymes split laminarin and its derivatives (chrysolaminarin), being the main storage carbohydrates of marine phytoplankton (Cox 1980) which, especially in periods of spring–summer bloom predominate in the crustacean's diet (Ligowski 1993).

Much less attention was payed to krill hydrolases of lipids though this constituent is abundant in marine plant and animal organisms found in *E. superba* stomachs (Marshall and Orr 1960, Marr 1962, Pavlov 1970, Baraškov 1972, Dorley 1977). The lipid content in diatoms ranges from 8 to 25% of organic matter and in spite of seasonal fluctuations is always lower in comparison to carbohydrate (30–70%) and protein (14–36%) contents (Baraškov 1972, Dorley 1977). On the other hand lipids and proteins constitute the main components of food of animal origin, the carbohydrate of which is usually transient (Mayzaud and Mayzaud 1981). The lipid content in organic matter of copepods (15–37%), juvenile forms and residual of matured specimens were found in *E. superba* stomach (Marr 1962, Pavlov 1970) equals to the protein contents during Antarctic autumn and winter (Marshall and Orr 1962).

Up to now no homogeneous lipases were isolated from Antarctic krill and characterized. Studies of Ellingsen and Mohr (1981) on *E. superba* autolysis *post mortem* indirectly revealed the high level of lipolytic activity in the crustacean, because of an intensive lipolysis of storage triacylglicerols and phospholipids just after initial stage of muscle proteins destruction which, as the authors presume, enables a contact of krill digestive lipolytic enzymes with their spatially separated endogenous substrates. However, the cited authors (Elling-

sen and Mohr 1981) have not performed direct assays of *E. superba* lipolytic activity. Results of such determinations, performed in our laboratory for various samples of Antarctic krill and its alimentary tract contents, are reported in the present paper.

Materials and methods

Biologial material: Samples of *E. superba* and its digestive tract contents, prepared during catching of Antarctic krill by workers of the Marine Fishery Institue in Gdynia and used for the studies are follows:

a) Krill frozen in blocks under the standard conditions, i.e. not later than 1–2 hrs after catching in a freezing canal at −28°C, in 1976, 1977, 1984, 1985 and 1986 .

b) Live specimens of *E. superba* taken from the net, immediately frozen at −40°C in sterile vials. Each portion of the frozen raw material was characterized with regard to the level of feeding, determined on the grounds of food intake in alimentary tracts of freshly caught specimens, according to a 5 point conventional biological scale (Wolnomiejski, Witek and Czykieta 1980). The samples were prepared from February to April 1970.

c) Liquid digesta obtained from each portion of raw material, described in (b), by centrifugation of live specimens in a basket centrifuge (380 × g, 1 mln). The supernatant was immediately frozen at −40°C in sterile vials.

d) acetone powders received from homogenates of freshly caught whole *E. superba* specimens, as well as homogenates of cephalothoraxes and abdomens, separated from freshly caught animals. The homogenates were treated (1:1 v/v, 5 minutes) with acetone cooled to −28°C and free from oxidation — reduction substances. The precipitates were dried under reduced pressure over P_2O_5 for 24 hrs and stored in sealed glass ampoules.

Samples b+d were prepared especially for the Institute of Technical Biochemistry of Technical University of Łódź. All the materials were stored at −30°C until use, no longer than 6–10 months.

Chemicals: 2-naphthyl derivatives of fatty acids: propionic, caprylic, myristic and stearic, tributyrylglicerol (tributyrin), fast blue B and methyl cellosolve (ethylene glycol monomethyl ether) were deliverd by Koch & Light (England). Polyvinyl alcohol was supplied by Serva (Germany). Other reagents were purchased from Polish Chemicals (Gliwice, Poland).

Preparing of enzymatic extracts: 1) 30 g of sample frozen krill was initially crumbled with scalpel and homogenized, at first in 100, and again in 25 cm^3 of 10 mM phosphate buffer pH 6.4, in Unipan 302 homogenizer at 10.000 rpm, each time 2 minutes at 0°C. After centrifugation (5,000 × g, 4°C, 20 min) of the successive homogenates, the supernatants were combined and used as an

enzymatic extract. 2) 1 g of acetone powder (in portions) was suspended in 10 mM phosphate buffer pH 6.4 (the total volume 25 cm^3) using Potter homogenizter and extracted for 15 minutes at 0°C. After centrifugation (5,000 × g, 4°C, 10 min) the sediment was removed, and the supernatant was used as an enzymatic extract.

Determination of protein: Protein content was determined according to Lowry *et al.* (1951) with bovine serum albumin as a standard.

Assays of lipolytic activity: Activity in hydrolysis of olive oil (40% emulsion in 2% polyvinyl alcohol) and tributyrin (0.1% emulsion in 40 mM phosphate buffer pH 6.4) was assayed by amperometric titration (Ardens 1974). 1 cm^3 of properly diluted enzymatic extract, 5 cm3 of substrate emulsion and 4 cm^3 of 40 mM phosphate buffer pH 6.4 were incubated for 10 minutes at 37°C. The reaction was stopped with 10 cm^3 of 96% ethanol and the samples was titrated with 5 mM NaOH up to pH 10.5. In control samples the emulsion of substrates and buffer were mixed with ethanol, and the enzymatic extract was added at the end.

The activity was expressed as nanomoles of fatty acids liberated from a substrate for 1 minute under reaction conditions (pH 6.4, 37°C, 10 min).

Lipolytic activity was also assayed against synthetic substrates, i.e. esters of 2-naphthol and fatty acids according to Seligman and Nachlas (1950) in modifican Breuil and Kuschner (1975). In this method, the incubation of a substrate with enzymatic extract in the presence of an emulsifier (sodium cholate) permits for total determination of lipases (EC 3.1.1.3) and carboxylesterases (EC 3.1.1.1), and in the absence of the emulsifier — only the latter enzymes. As the result of this, the lipolytic activity is precisely determined. 2.8 cm^3 of 40 mM phosphate buffer pH 6.4, 0.5 cm^3 of 0.23 M sodium cholate solution (or water in carboxylesterase assay) and 0.2 cm^3 of 2.5 mM substrate solution in methyl cellosolve were incubated for 10 minutes at 37°C, and 0.2 cm^3 of enzymatic extract was subsequently added. The samples were incubated for 10 minutes at 37°C. 2-naphthol liberated was determined colorimetrically in reaction with tetrazolium derivative of o-dianisidine (fast blue B) according to Breuil and Kuschner (1975). The control samples were prepared for thermally inactivated enzymatic extracts.

The activity was expressed as nanomoles of 2-naphthol (equals to nanomoles of fatty acids) liberated from a substrate for 1 minute under assay conditions (pH 6.4, 37°C, 10 min).

Other methods: Optimal pH in hydrolysis of 2-naphthyl myristate was estimated under standard conditions (37°C, 10 min) using 40 mM Britton-Robinson buffer solution with pH in the range 2.6–10.0. Optimal temperature of *E. superba* lipases was found out for 10 minutes reaction of 2-naphthyl myristate hydrolysis, performed at different temperatures in the range 4–60°C at pH 6.4. For studies of thermostability, the samples of

enzymatic extract containing 0.25 mg of protein were incubated up to 45 minutes at temperatures in range 4–60°C. The residual activity against 2-naphthyl myristate was determined after 5, 15, 30 and 45 minutes under standard condition (pH 6.4, 37°C, 10 min). Michaelis constants (K_m) for reactions of 2-naphthyl myristate and tributyrin hydrolysis were estimated at pH 6.4 and 37°C using substrate concentrations in the range, respectively, $2.8-28\times10^{-3}$ M and $0.5-10.0\times10^{-3}$ M. The values of K_m and maximal reaction rate (V_{max}) were defined according to Lineweaver-Burk's method (1934).

Results and discussion

The lipolytic activity against 2-naphthyl myristate of frozen *E. superba* samples, prepared in conditions which considerably limited the possibility of the animal's autolysis before freezing (live specimens were frozen immediately after capture), is correlated to a feeding level of the animals present in the samples (Tab. 1). Both specific and total activities were 3.5–4.5 fold higher in samples of the crustaceans with high food intake (feeding level lower to 4 or higher) in comparison to the starving ones (feeding level lower than 3, Tab. 1). The relationship was much stronger expressed for liquid digesta from krill samples examined, prepared from fresh animals immediately after capture, under relatively mild conditions. The specific activity of lipase in the latter material, obtained from specimens with full digestive tracts, was about 9 fold higher in comparison to liquid digesta derived from animals with empty alimentary canals (Tab. 1). The results confirm a digestive character or the activity determined.

Krill liquid digesta separated by very short, filtrate centrifugation of fresh crustaceans (1 minute, 380 × g) amounted to about 23% of their mass (K. Kołodziej, *pers. commun.*). Taking into consideration lipolytic activity (535–563 units/g of dry mass) and a solid matter content (23.2 to 27.1%) in the frozen crustaceans used for assays, one might expect that the lipolytic activity in samples of liquid digesta would be equal to 28–35 units/cm^3. However, it is 6 fold higher, on the average (Tab. 1). The fact might point to a presence of a substance, probably of a tissue origin, which inhibits lipolytic activity in enzymatic extracts obtained from homogenates of whole animals.

Table 2 presents the results of our preliminary studies on specificity of lipolytic enzymes present in feeding specimens of *E. superba*. Synthetic substrates, i.e. 2-naphthyl derivatives of fatty acids with different chain lengths and tributyrylglycerol, as well as olive oil as a natural substrate, were used for assays. The experiments allowed to estimate a carboxylesterase activity which is independent on the presence of an emulsifier (sodium cholate) in reaction mixtures which, on the other hand, is necessary for the activity of lipases, i.e. acylhydrolases of triacylglycerols (EC 3.1.1.3). The carboxylesterase activity

Table 1.

Lipolytic activity of frozen krill (samples with different food intake, frozen immediately after catching at −40°C) and its digestive tract contents.

The activity was assayed with 2-naphthyl myristate as a substrate according to methods

Average results calculated on the basis of 5 assays performed for 4 different portions of each krill sample are presented

Sample number[1]	Day of krill catching[1]	Region of catching[2]	Food intake[2]	Activity			
				Extracts from the whole krill		Liquid digesta	
				units/mg of protein	units/g of krill dry mass	units/mg of protein	units/cm³
1	February 6th	60'55:S 40'04:W	4.02	3.7	535	15.0	219.5
2	February 13th	59'30:S 44'11:W	3.95	3.0	534	14.0	171.1
3	February 25th	61'03:S 54'26:W	4.27	3.5	563	17.00	194.0
4	March 28th	54'02:S 35'41:W	2.53	0.7	119	1.8	48.0
5	April 21st	54'32:S 35'36:W	2.46	0.8	180	1.5	50.3

1) Sample of type b (see: Biological Materials)

2) The data of K. Kołodziej from the Marine Fishery Institute in Gdynia

Table 2.

Lipolytic (L) and esterolytic (E) activities of frozen krill extracts (sample no 1, table 1) in the hydrolysis of synthetic substrates and the olive oil
Average results of 5 activity assays in 5 samples of the frozen crustacean, caught in February 6th, 1979 (table 1) are presented

Substrate (kind of fatty acid)	Kind of activity	Activity	
		units/mg of protein	units/g of krill dry mass
2-Naphtyl propionate (C_3, saturated)	E	106.6	15.440
2-Naphtyl caprylate (C_8, saturated)	L	0.7	100
	E	6.4	930
2-Naphtyl myristate (C_{14}, saturated)	L	3.7	535
2-Naphtyl stearate (C_{18}, saturated)	L	1.9	276
Olive oil (C_{18}, unsaturated)	L	9.4	1.360
Tributyrylgylcerol (C_4, saturated)	L	59.1	8.570

was observed against 2-naphthyl derivatives of propionic and caprylic acids, only. It was more than 10 times higher towards the ester of C_3 fatty acid than towards the C_8 one (Tab. 2). However, the krill extracts carboxylesterase does not split esters of long chain fatty acids, i.e. C_{14} and C_{18}, because in the absence of sodium cholate they are not hydrolysed. It should be stressed that the specific activity of carboxylesterase in the sample of feeding krill, caught during Antarctic summer (February 6th, 1979) against 2-naphthyl propionate was very high (106.5 units/mg of protein) and comparable to the laminarinase activity (95.8 units/ng of protein), since the meaning of the units is comparable (Turkiewicz 1991).

From all applied for studies, synthetic substrates which were esters of 2-naphthyl and fatty acids, lipases from *E. superba* extracts prefer 2-naphthyl myristate (C_{14}). Their activity towards this substrate is 2-fold higher than against the derivative of stearic acid (C_{18}). On the other hand, their activity against 2-naphthyl caprylate is inconspicuous (Tab. 2). It is 10-fold lower than the carboxylesterase activity against the same substrate. The comparison of lipolytic activities towards 2-naphthyl stearate and olive oil which are esters of C_{18} fatty acids, proves that krill enzymes markedly prefer esters of unsaturated fatty acids as substrates, because their activity towards olive oil is 6-fold higher than against the second substrate (Tab. 2). Probably, this preference results from an ascendancy of lipids containing polyunsaturated fatty acids (PUFAs) in organism living at low temperatures and consumed by krill. For example, triacylglycerols which are derivatives of C_{18} and C_{20} PUFAs are abundant in marine diatoms. Similar PUFAs were found in Dinoflagellata (18:4, 20:5, 22:6) (Marshall and Orr 1960, Kates and Volcani 1966, Baraškov 1972, Opute 1974) which krill also feeds on.

Tributylglycerol which is reckoned as one of the most accessible substrates of some lipases (Vorderwülbecke, Kieslich and Erdermann 1992) is decomposed

by krill extracts with the highest actitivy which is of the same range as their proteolytic activity towards denatured haemoglobin (Turkiewicz *et al.* 1986) and the laminarinase one (Turkiewicz, Galas and Zielińska 1985).

Table 3 presents the results of lipolytic activity determinations in *E. superba* samples frozen under the standard conditions, performed for several years. Although an average feeding level of frozen specimens has not been estimated for these samples, we might presume that it was high, on the grounds of their activities against tributyrin and olive oil. Results of the determinations suggest that Antarctic krill lipases are resistant to the crustacean's proteinases attack, since their activities in samples stored on a board even for 1 – 2 hours after capture (Tab. 3) and in samples of krill with high food intake, frozen immediately after catching, are similar (Tab. 2). Our previous studies confirmed that other *E. superba* digestive hydrolases, as for example amylases (Turkiewicz and Kalinowska 1993) and β-1,4-xylanases (Turkiewicz 1995) are not so resistant, because their activities are much more higher in the crustacean's samples frozen immediately after capture, and only an inhibition of krill serine proteinases (for example with PMSF) stabilizes their activities during storage and purification on the extracts.

Table 3.

Lipolytic activity of krill samples frozen under the standard conditions (samples of type a, see: Biological material)
Results are average values of 10 assays carried out for 5 samples of each material

Year of catching	Activity			
	Tributyrylglycerol		Olive oil	
	units/mg of protein	units/g of krill dry mass	units/mg of protein	units/g of krill dry mass
1976	101.0	15.420	12.1	1.857
1977	74.3	13.050	10.6	1.770
1984	86.0	15.730	8.5	1.320
1985	85.5	12.660	11.4	1.683
1986	81.0	13.770	8.5	1.440

It was testified that majority of Antarctic krill digestive enzymes is secreted by parts of alimentary canal localized in cephalothorax (Ellingsen 1982, Turkiewicz 1991) which among others contain a hepatopancreas, reckoned as the main *E. superba* digestive organ (Mauchline 1980).

Determination of lipolytic acitvity in extracts from acetone powders derived from homogenates of whole krill, its cephalothorax and abdomen (Tab. 4) supplied initial data about lipases location in *E. superba* . The highest activity towards tributyrin and olive oil was detected in cephalothorax, however, it was also marked in abdomen, containing hind intestine with a gland structure (Mauchline and Fisher 1969). One may presume that *E. superba* possesses two

Table 4.

Localization of *E. superba* lipolytic enzymes

Activity was assayed according to methods in the enzymatic extracts obtained from preparations precipited with acetone from the homogenates of whole krill specimens, their cephalothoraxes and abdomens

Average results of 5 assays performed for 2 samples of each extract are presented

Enzymatic extract from	Activity			
	Tributyrylglycerol		Olive oil	
	units/mg of protein	units/g of krill dry mass	units/mg of protein	units/g of krill dry mass
whole krill	92.8	19.480	16.9	3.550
cephalothorax	134.4	26.880	24.9	4.980
abdomen	37.1	7.420	8.2	1.640

kinds of lipases including intestine enzyme, similarly to vertebrates. If its specificity resembles a vertebrate intestine lipase, the *E. superba* enzyme should be more active against monoacylglycerols, not applied for our studies.

Lipases from Antarctic krill extracts exhibit the highest activity at pH 6.4 and about 37°C for 10 minutes against 2-naphthyl myristate (Figs. 1 and 2a) and tributyrylglycerol (results not presented). They exhibit as much as 10% of maximal activity at 4°C (Fig. 2a), i.e. at the temperature similar to the temperature of *E. superba* habitat. The enzymes are highly termolabile since they preserve total activity up to 45 minutes at temperatures not exceeding 35°C

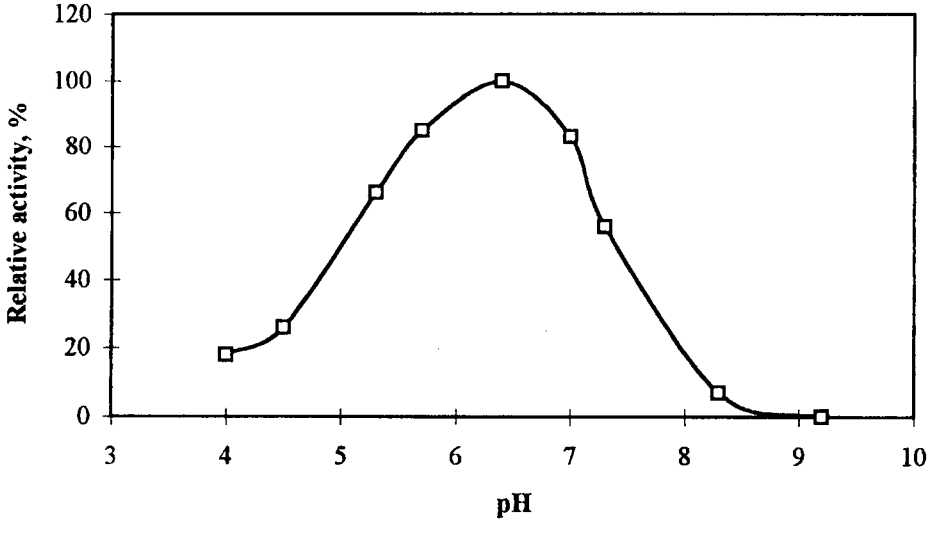

Fig. 1. Effect of pH on lipolytic activity of *E. superba* enzymatic extracts. Maximal activity (4.2 units/mg of protein at pH 6.4 in 2-naphthyl myristate hydrolysis) was considered to be 100%

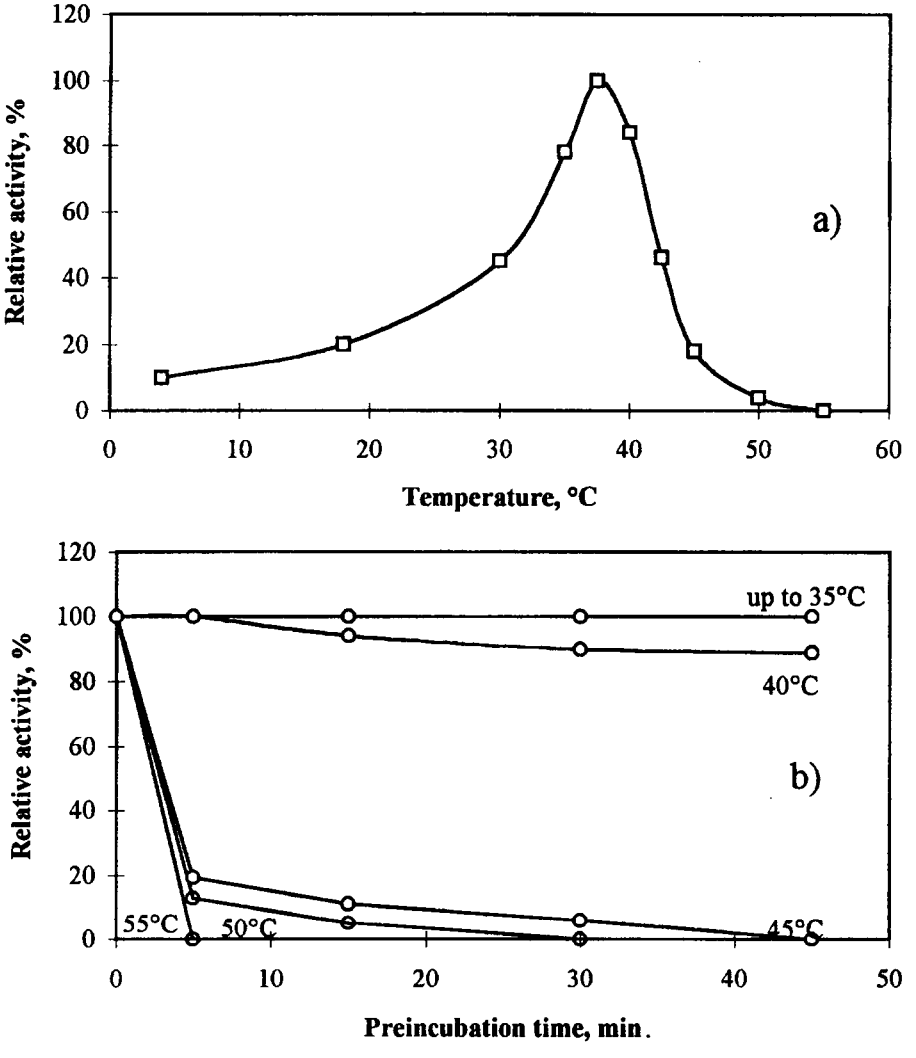

Fig. 2. Effect of temperature on lipolytic activity (a) and stability (b) of *E. superba* enzymatic extracts. (a) Maximal activity (4.2 units/mg of protein at 37°C in 2-naphthyl myristate hydrolysis) was equivalent to 100%

(b) Activity of extracts not incubated prior to assays was considered to be 100%

Table 5.

Kinetics parameters of *E. superba* lipolytic enzyme.
Assays were performed according to methods with enzymatic extract obtained from krill samples No 1 (sample of type b, see: Biological material)

Substrate	K_m	V_{max}
	mM	units/mg of protein
2-Naphthyl myristate	15.51	7.5
Tributylglicerol	1.12	159.00

(Fig. 2b). At 40°C the decrease of the activity is still small (11% for 45 minutes), but at higher temperatures their activity decreases dramatically, especially for the first 5 mintues of heating. The total loss of lipolytic activity is observed at 55°C for 5 minutes. Such a low thermostability was also testified for *E. superba* trypsin-like proteinases (Osnes and Mohr 1985), a collagenolytic serine proteinase (Turkiewicz 1991) and the crustacean's β-1,4-xylanases (Turkiewicz 1995). Krill lipases show a low affinity towards 2-naphthyl myristate ($K_m = 1.55 \times 10^{-2}$ M; Tab. 5) and 10-fold higher towards tributyrin (1.12×10^{-3} M).

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Streszczenie

W próbach antarktycznego kryla zamrażanych w różnych warunkach (w −28°C w ciągu 1–2 godzin po wyłowieniu i w −40°C natychmiast po wyłowieniu), a także w treści przewodu pokarmowego, wypreparowanej ze świeżych zwierząt, oznaczono aktywność lipolityczną stosując substraty syntetyczne (tributyryloglicerol oraz estry 2-naftolu i kwasów tłuszczowych C_3, C_8, C_{14} i C_{18}) oraz olej z oliwek. Stwierdzono, że aktywność ta jest 3.5–4.5-krotnie wyższa w skorupiakach żerujących niż u zwierząt z pustym przewodem pokarmowym (TAb. 1). Lipazy kryla z najwyższą aktywnością hydrolizują tributyryloglicerol (Tab. 2), względem którego wykazują wysokie powinowactwo (K_m=1,12 mM), o rząd wielkości wyższe, aniżeli względem 2-naftylomirystynianu (K_m=15,51 mM, Tab. 5). Preferują jako substraty estry nienasyconych kwasów tłuszczowych, w rozkładzie których ich aktywność jest ok. 5-krotnie wyższa, aniżeli w hydrolizie estrów nasyconych kwasów tłuszczowych o tej samej długości łańcucha (Tab. 2). Lipazy *E. superba* wykazują maksymalną aktywność w pH 6.4 i 37°C (Rys. 1 i 2a). Charakteryzuje je wysoka termolabilność; pełną aktywność zachowują w temperaturach do 35°C w ciągu 45 minut, w 55°C inaktywują sie całkowicie w ciągu 5 minut (Rys. 2b). Stwierdzono, ze główna część aktywności lipolitycznej jest zlokalizowana głowotułowiu kryla, jednakże ekstrakty enzymatyczne odwłoka wykazują również znaczną aktywność w hydrolizie triacylogliceroli (Tab. 4). Może to dowodzić, że *E. superba*, podobnie jak kręgowce, wytwarza dwa rodzaje lipaz, w tym enzym jelitowy. Porównanie aktywności lipolitycznej w krylu zamrażanym natychmiast i w ciągu 1–2 godzin po wyłoweiniu (Tab. 1 i 2) wskazuje, że lipazy kryla są stosunkowo odporne na atak jego protein.