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Immobilization of Lipase Inhibitor on the Biopolymers from *Agaricus bisporus* Cell Walls

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Summary

One of the methods for curing obesity is the inclusion of some substances with the antilipase activity in the diet and thus reducing the uptake of fat components from food. The aim of this research is to provide a stabilized form of lipase inhibitor by immobilization of enzyme on the biopolymers from *Agaricus bisporus* cell walls. The phenolic compounds extracted from the rapeseed were considered as the lipase inhibitor. The activity of the inhibitor was considerably reduced in the gastric juice, as well as at temperatures above 37 °C and during its storage, which determined the suitability of the inhibitor for stabilization on the matrix. The effectiveness of the phenolic compound stabilization was investigated by means of immobilization on the biopolymers from *Agaricus bisporus* cell wall matrix. The biopolymers used were β-glucan, chitin, melanin and proteins. A number of samples, which differed both in the content of the inhibitor (from 1 to 16 %) and in the ratio of biopolymers in the matrix composition, was obtained. The conditions of immobilization (temperature, duration of the process) were also varied. The expediency of obtaining the sample with the inhibitor content of 12 % and matrix containing 47.9 % of glucan, 18.8 % of chitin, 18.8 % of melanin and 11.1 % of proteins was shown. The best immobilization was carried out at 20–25 °C for 30 min. Thermal analysis and infrared spectroscopy data confirmed that immobilization of the lipase inhibitor on the matrix was due to the hydrogen bonds. The immobilized inhibitor had higher pH stability and higher thermal stability than the original one. The remaining activity of the immobilized inhibitor was higher than the original one after incubation in the gastric acid and bile. The immobilized inhibitor was characterized by a low loss of activity after 12 months of storage.

Key words: lipase inhibitor, biopolymers, immobilization, mushroom, rapeseed

Introduction

In recent decades, the population of many countries has faced the problem of obesity (*1,2*). For a long time we believed that it did not have a serious impact on the human health. However, at the turn of the 21st century, the World Health Organization has changed its opinion on this problem. The results of medical studies have shown that the chronic form of obesity is a disease associated with insulin-independent diabetes, hypertension, atherosclerosis, gallstone disease and some malignant diseases. In addition, the medical prognosis is unfavourable, as the number of patients that suffer from chronic obesity is increasing; every second inhabitant of our planet will face this problem in 10 years (*2-4*).

In order to fight against this disease, scientists have developed the number of drugs. However, a detailed study of their impact on the human body revealed that only one of them is safe to use. It is a drug called Xenical (orlistat). This product is obtained by hydrogenation of lipstatin (*5-7*).

Orlistat inhibits the lipase activity in the human body. It reduces decomposition of exogenous fats and facilitates their excretion from the body. Thus, it shows antilipase activity and reduces the digestibility of fat. However, in some cases, its application is accompanied by a violation of the

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qualitative and quantitative composition of the intestinal microflora. This, in turn, can disrupt the balance of the entire microbial-intestinal tissue complex (*8*).

Inhibitors of pancreatic lipase derived from the plant sources have no side effects. They do not cause addiction or allergies and they are nontoxic, in contrast to microbial inhibitors (*9*). Therefore, the development of antilipase drugs produced from plant sources is relevant.

Some existing dietary supplements that contain plant components can inhibit the lipase action. They are manufactured in Indonesia and include products from leaves or extracts of *Guazuma ulmifolia* and Brassicaceae family plants. They show antilipase activity due to the phenolic compounds. When coffee beans and leaves of black and green tea are included in the dietary supplements, the alkaloids and the catechins of the raw materials are the active compounds inhibiting lipase (*10-13*). However, application of some dietary supplements that contain the sources of lipase inhibitors listed above has several disadvantages. They have some antinutrients, such as the cyanogenic glycosides and alkaloids (*14,15*).

Among the European plants, the plant families Leguminosae, Cruciferae, Papaveraceae, Apiaceae and Brassicaceae have the highest antilipase activity (*10*). However, due to the climate conditions, plants that grow in Europe have lower antilipase activity than Indonesian ones. The rapeseed, which belongs to the family Brassicaceae, is the prospective source of the compounds with antilipase activity. The advantage of this culture as a raw material for producing lipase inhibitors is that the rapeseed is one of the most productive oilseed crops in the world. Therefore, in recent years, its cultivation area had increased. Furthermore, not only rapeseed but also some by-products of its processing can be used as a source of phenolic compounds.

The phenolic acids, as well as the soluble and insoluble tannins are the main carriers of the antilipase activity in the rapeseed (*16,17*). The phenolic acids derived from benzoic and cinnamic acids occur in the rapeseed in the free and bound forms. The sinapic acid and its derivative sinapine (choline ester of 3,5-dimethoxy-4-hidroxycinnamic acid) predominate among the phenolic acids in the rapeseed (*18*).

Direct addition of rapeseed into dietary supplements as a source of the lipase inhibitor is not recommended, due to its low content of phenolic compounds and antinutrient substances, particularly erucic acid and glycosinolates (*10*). The rapeseed phenolic compounds extracted from the raw materials are unstable and they rapidly lose their antilipase activity. Therefore, it is important to obtain the type of the inhibitor that will be resistant to external influences.

Immobilization is one of the well-known methods of stabilizing biologically active substances (*19,20*). Considering that the purpose of immobilization is to obtain the phenolic compounds that are resistant to the environmental influence, we regarded biopolymers from *Agaricus bisporus* cell walls as a matrix (*21*), chosen for their antioxidant and antacid properties. The first goal was to create the prerequisites for preserving the antilipase activity of the inhibitor during storage and the second to protect it against the influence of the acid medium during passage through the stomach.

The aim of this research is to obtain the stabilized form of the lipase inhibitor by its immobilization on the biopolymers from *Agaricus bisporus* cell walls.

Materials and Methods

Materials

All reagents and standards were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). *Agaricus bisporus* was purchased at the farm that cultivates mushrooms, Geleka-M Company (Odessa, Ukraine). The fruiting bodies were cleaned, dried and milled. The rapeseed (*Brassica napus*) was obtained from List Company (Kiev, Ukraine). The drug Xenical was from Roche (Basel, Switzerland).

Preparation of matrices for inhibitor immobilization

During the experimental research, samples of *Agaricus bisporus* biopolymers were used as a matrix for the inhibitor immobilization. Solid residues were obtained after the sequential treatment of the mushroom with three extraction agents: boiling water for 90 min, 3.7 % HCl at 25 °C for 150 min and 3 or 7 % NaOH at 98 °C. The duration of the alkaline treatment was 60 or 300 min (Table 1). The solid residue was washed until the neutral pH was obtained and then it was freeze dried. The matrix without melanin was obtained by the additional treatment of sample 5 (Table 1) with 8 % H_2O_2 solution for 60 min at room temperature.

Table 1. Conditions used for obtaining samples of matrices

Sample		Water	*w*(HCl) =3.7 %	*w*(NaOH)/%	*w*(H_2O_2)/%
1	*t*/°C	100	25	3	
	Time/ min	90	150	98 60	–
2	*t*/°C	100	25	3	
	Time/ min	90	150	98 300	–
3	*t*/°C	100	25	7	
	Time/ min	90	150	98 60	–
4	*t*/°C	100	25	7	
	Time/ min	90	150	98 300	–
5	*t*/°C	100	25	7	8
	Time/ min	90	150	98 60	60

Determination of chemical composition of matrices

Matrices were treated with 72 % H_2SO_4 at room temperature for 150 min. Water was added to decrease acid volume fraction to 5 %. Then the matrices were hydrolysed for another 300 min at the boiling temperature (*22*). Melanin content was determined by UV-Vis spectrophotometer (UV-2401 PC; Shimadzu Corp., Kyoto, Japan) at the wavelength λ=490 nm (*23*). The polysaccharide content (except chitin) was determined by the anthrone method. A volume of 4 mL of anthrone reagent (1 mg of anthrone

per 1 mL of H_2SO_4) was added to 0.5 mL of working solution, the mixture was bathed for 15 min, cooled to the room temperature, and the absorbance was measured by UV-Vis spectrophotometer (UV-2401 PC; Shimadzu) at λ=670 nm (*24*). The glucosamine content was evaluated with 3-methyl-2-benzothiazolone hydrazone hydrochloride according to Lane Smith and Gilkerson (*25*). To 1 mL of the sample solution, 1 mL of 5 % $KHSO_4$ and 1 mL of 5 % $NaNO_2$ were added. The mixture was shaken at 5000 rpm for 15 min, then 1 mL of 12.5 % $NH_4SO_3NH_2$ was added and shaken for 5 min. A volume of 1 mL of 0.5 % 3-methyl--2-benzothiazolone-hydrazone-hydrochloride was added and the mixture was left to settle for 60 min. Then 1 mL of 0.5 % $FeCl_3$ was added and the absorbance was measured by UV-Vis spectrophotometer (UV-2401 PC; Shimadzu) at λ=650 nm.

The monosaccharides from the hydrolysates were converted into their corresponding alditol acetates (*26*) and identified by gas-liquid chromatography (Hewlett-Packard 5890 A chromatograph; Hewlett-Packard, St. Louis, MO, USA) with a flame ionization detector and integrator 3393 A with an Ultra-1 capillary column (25 m×0.2 mm) at temperatures from 175 to 270 °C and a rate of 10 °C/min in a stream of nitrogen.

The content of nitrogen in protein was estimated as the difference between the total nitrogen determined by Kjeldahl method (*27*) and nitrogen in chitin. The content of nitrogen in chitin was estimated by dividing the chitin content by coefficient 6.89 (*28*). The total protein content was calculated by multiplying the protein nitrogen by 6.25 (*27*).

Inhibitor preparation

The precrushed and defatted rapeseed was treated with 96 % ethanol at 30 °C for 30 min. The ethanol extract that contained the phenolic compounds was separated by centrifugation at 5000 rpm (centrifuge model OS-6MC; Dastan, Kazakhstan). From this extract the ethanol was removed by vacuum distillation. The resulting residue was dissolved in water to obtain the mass per volume ratio of the inhibitor in the solution from 0.17 to 4.77 % (Table 2).

Determination of chemical composition of the inhibitor

The content of phenolic compounds was determined by Folin-Ciocalteu method (*29*). The composition of phenolic compounds was determined by thin layer chromatography (TLC) on CAMAG TLC Scanner 4 (Muttenz, Switzerland) apparatus equipped with automatic TLC

Table 2. Conditions used for preparation of the immobilized inhibitor

Sample of immobilized inhibitor	*w*(inhibitor)/%	Initial components			
		Matrix		Inhibitor solution	
		Sample no.	*m*/g	*w*/%	*V*/mL
1’	1	1	0.99	0.25	4
1’	4	1	0.96	1.04	4
1’	7	1	0.93	1.88	4
1’	10	1	0.90	2.78	4
1’	12	1	0.88	3.41	4
1’	14	1	0.86	4.07	4
1’	16	1	0.84	4.77	4
2’	1	2	0.99	0.25	4
2’	4	2	0.96	1.04	4
2’	7	2	0.93	1.88	4
2’	10	2	0.90	2.78	4
2’	12	2	0.88	3.41	4
2’	14	2	0.86	4.07	4
2’	16	2	0.84	4.77	4
3’	1	3	0.99	0.17	6
3’	4	3	0.96	0.69	6
3’	7	3	0.93	1.25	6
3’	10	3	0.90	1.85	6
3’	12	3	0.88	2.27	6
3’	14	3	0.86	2.71	6
3’	16	3	0.84	3.17	6
4’	1	4	0.99	0.17	6
4’	4	4	0.96	0.69	6
4’	7	4	0.93	1.25	6
4’	10	4	0.90	1.85	6
4’	12	4	0.88	2.27	6
4’	14	4	0.86	2.71	6
4’	16	4	0.84	3.17	6

sampler 4, twin trough chamber (20 cm×10 cm), chromatogram immersion device III, TLC plate heater III, automatic development chamber ADC2 and visualizer. The mobile phase was benzene/ethanol/acetic acid in a volume ratio of 90:16:9. The phenolic compounds were visualized by spraying the plates with water solution of ferrum chloride and ferrocyanide potassium (*30*). The sinapine and sinapic acid were identified by comparing the R_f value (distance eluent travels was 10 cm) of the standard substances. The quantitative content of the sinapine and sinapic acid was perfomed by CAMAG TLC Scanner 4 apparatus. Additionally, the phenolic compounds were separated by TLC as described above, but the plate was not sprayed with water solution of ferrum chloride and ferrocyanide potassium. The spots of synapine and synapic acid were determined from the R_f value. These phenolic compounds were extracted with 96 % ethanol at 30 °C for 30 min and their antilipase activity was determined (*31*).

Determination of lipase and antilipase activity

The lipase activity was determined according to the method of Jagdish *et al.* (*32*) with some modifications. The lipase (from porcine pancreas) was prepared at the concentration of 2.5 mg/mL. The substrate was prepared by mixing 100 mL of olive oil and 150 mL of 2 % polyvinyl alcohol.

The experimental sample was prepared as follows: 5 mL of the substrate and 4 mL of phosphate-citrate buffer (pH=7) were placed in the flask and kept at 37 °C for 15 min. After that, 1 mL of the enzyme solution was added to the mixture and mixed thoroughly. The resulting mixture was kept at 37 °C for 60 min. Then, 30 mL of 90 % ethanol were added to the mixture immediately to stop the reaction. Using phenolphthalein solution as the indicator, the solution was titrated with 0.05 M NaOH solution until a pink colour was obtained.

The control sample was prepared as follows: the mixture of the substrate and phosphate-citrate buffer (pH=7) was kept at 37 °C. Then 30 mL of 90 % ethanol and 1 mL of the enzyme solution were added and the mixture was titrated immediately.

The lipase activity (in U/g) was calculated according to the formula:

$$\text{Lipase activity}=(V_1\text{-}V_2)\cdot V_3\cdot 50/\gamma \qquad /1/$$

where V_1 is the volume of 0.05 M NaOH solution (in mL) used for the experimental sample titration, V_2 is the volume of 0.05 M NaOH (in mL) used for the control sample titration, V_3 is the titter of 0.05 M NaOH (in mL) and γ is the concentration of the enzyme in the reaction mixture (in g/mL).

The lipase activity in the presence of the inhibitor was assayed as described above. A distinction was that the inhibitor containing 0.5–8.0 mg of the phenolic compounds was added to the mixture at once after adding the enzyme solution (in experimental and control samples), and then the mixture was stirred thoroughly.

The antilipase activity of the inhibitor and Xenical was determined as the difference between the values of the lipase activity in the enzyme solution and the lipase activity in the enzyme solution in the presence of 20 mg of Xenical.

Determination of inhibitor properties

For determination of the pH stability of the inhibitor, 6 mg of the inhibitor were incubated in 100 mL of the medium with different pH values (HCl-KCl, pH=2; citrate buffer, pH=5; sodium phosphate buffer, pH=7 and Tris-HCl, pH=9) for 0–360 min at (37±2) °C. The samples were taken every 60 min, the pH of the mixture was adjusted to pH=7, and the antilipase activity of the samples was determined.

To determine the thermal stability of the inhibitor, 6 mg of the inhibitor were incubated at (20±2), (37±2) and (65±2) °C for 0–360 min. The samples were taken every 60 min, their temperature was adjusted to (37±2) °C, and the antilipase activity of the samples was determined. Remaining antilipase activity in that test, as well as those described below, were estimated as the percentage of the initial value of the antilipase activity of the inhibitor.

To predict the preservation of antilipase activity in the gastrointestinal tract, 6 mg of inhibitor were placed in a flask and incubated in 100 mL of gastric juice at (37±2) °C for 180 min. After that, the pH in the flask was adjusted to pH=8 and 100 mL of the natural bile were added. The mixture was incubated at (37±2) °C for 180 min. The inhibitor was kept in the gastric juice and bile, the samples were taken every 60 min, the pH of the medium was adjusted to pH=7 and the antilipase activity of the inhibitor was determined.

To determine the changes in antilipase activity during storage, 6 mg of the inhibitor were incubated for 7 days at (20±2) °C and relative humidity of 75 %. The antilipase activity was determined after 6 h and then after 1, 3, 5 and 7 days.

Preparation of the immobilized lipase inhibitor

To immobilize the phenolic compounds, the biopolymer matrix was impregnated with their solution. The content of the inhibitor in samples 1'–4' varied in the range from 1 to 16 % (Table 2). For preparing samples 1' and 2', 4 mL of the inhibitor solution (with a mass per volume ratio of 0.25 to 4.77 %) were mixed with 1 g of matrices 1 and 2. For preparing samples 3' and 4', 6 mL of the inhibitor solution (with a mass per volume ratio of 0.17 to 3.17 %) were mixed with 1 g of matrices 3 and 4 (Table 2). The immobilization temperature was varied from 20 to 40 °C during 50 min of immobilization, and the immobilization time was varied from 10 to 50 min at the temperature of 25 °C.

Determination of antilipase activity of the immobilized inhibitor

The antilipase activity of the immobilized inhibitor was determined as the difference between the lipase activity value of the enzyme solution (as described above) and the lipase activity value of the enzyme solution in the presence of 50 mg of samples 1'–4' (all containing 12 % of the inhibitor). The lipase activity of the enzyme solution in the presence of samples 1'–4' was determined in the same way as described for the original inhibitor. The antilipase activity of the immobilized inhibitor was calculated as a percentage of the activity of the original inhibitor.

Determination of immobilized inhibitor properties

The thermal and pH stability of the immobilized inhibitor (samples 1'–4' with 12 % of the inhibitor), as well as its remaining activity in the gastrointestinal tract were determined in the same way as the original inhibitor. To determine the pH stability and thermal stability, 50 mg of samples 1'–4' were used. For determination of the changes in the antilipase activity during storage, 50 mg of samples 1'–4' were kept for 12 months at (20±2) °C and air humidity of 75 %. Every two months, their antilipase activity was determined. The remaining antilipase activity of the immobilized inhibitor in these tests was calculated as a percentage of the activity of the original inhibitor.

Infrared spectroscopy and thermal analysis of the immobilized inhibitor

Infrared (IR) spectroscopic study of the original inhibitor and samples 1'–4' with 12 % of the inhibitor was carried out using the FTIR-8400S Fourier transform IR spectrophotometer (Shimadzu Corp.) in the range of 4000–400 cm^{-1}. The IR spectra were compared according to Bikales and Segal (*33*). Differential thermal analysis of the mixture of the inhibitor and samples 1–4 and 1'-4' with 12 % of inhibitor was carried out on the MOM derivatograph Q-1500 D (Paulik-Paulik-Erdey, Budapest, Hungary) (*34*). The samples were placed in an alumina cup and heated from 20 to 900 °C at a heating rate of 5 °C/min in a nitrogen atmosphere. The flushing gas was passed through dry nitrogen at a rate of 100 mL/min.

Statistical analysis

The statistical analysis of the data was carried out by the dispersion and correlation analysis (*35*). The statistical differences were considered significant at $p<0.05$. The results are expressed as mean values±standard deviation (S.D.).

Results and Discussion

Inhibitor composition and its properties

The rapeseed contained 7.2 mg/g of the phenolic compounds extracted with 96 % ethanol. Six phenolic compounds in the extract were found by TLC method. Sinapine (5.9 mg per g of seed) and sinapic acid (0.7 mg per g of seed) were dominant among them. The antilipase activity of the extract was 29 800 U per g of phenolic compounds, including the activity of sinapine, which was 18 200 U per g of phenolic compounds, and the activity of sinapic acid, which was 9700 U per g of phenolic compounds. The antilipase activity of the phenolic compounds isolated from the rapeseed was comparable to that of the drug Xenical (9800 U/g). For this reason, it was expedient to obtain the dietary supplements with antilipase activity.

The lipase interacts with the lipids in the intestine (*17*), so the activity of the inhibitor must move along the digestive tract through the stomach (pH~2.0) and the intestine (pH~9.0). Thus, the inhibitor stability under these conditions was an important factor.

As it is shown in Fig. 1, at pH=2 the antilipase activity decreased after incubation for 60 min, then after 180 min it decreased 1.6 times and finally after 360 min only 40.2 % of the initial inhibitor antilipase activity remained. At pH=5 the stability of the phenolic compounds was slightly

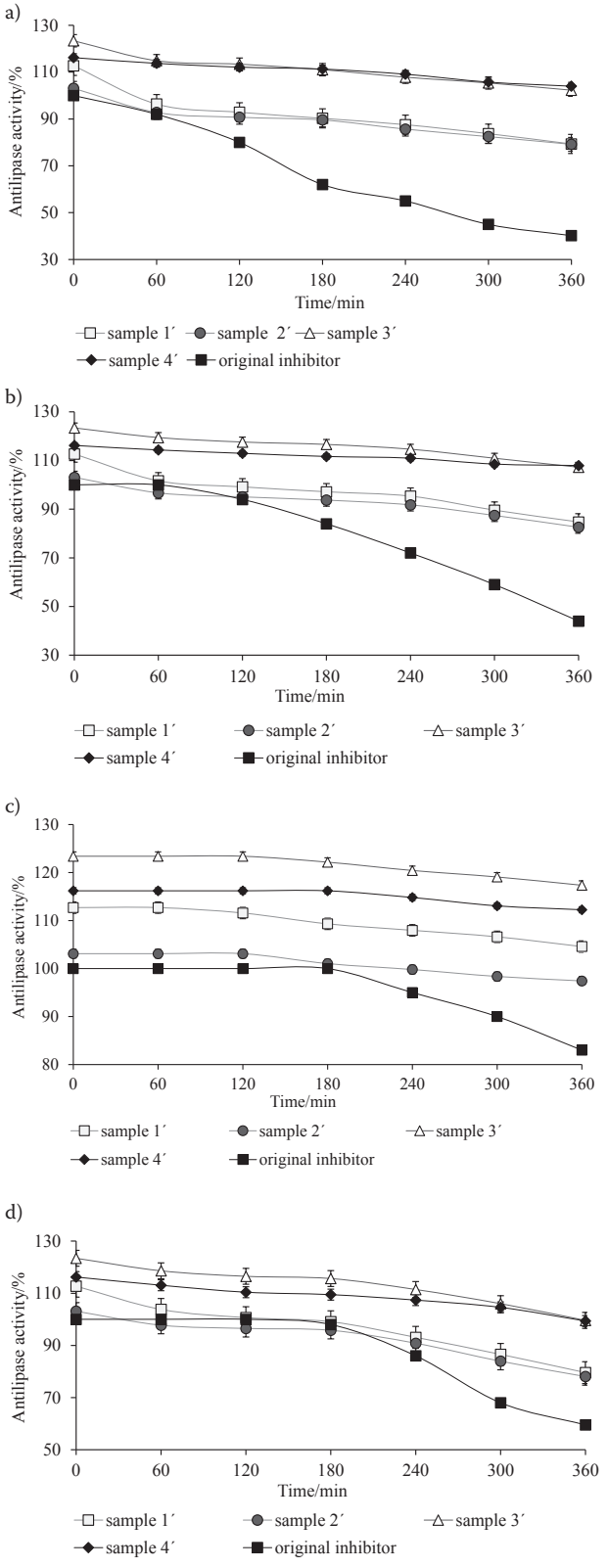

Fig. 1. The stability of samples 1'–4' at pH values of: a) 2.0, b) 5.0, c) 7.0 and d) 9.0

higher. They were stable at pH=7 and 9 for 180 min. However, when the incubation time increased, the antilipase activity significantly decreased. Thus, it was necessary to use aqueous solution of phenolic compounds (pH=7) for the immobilization on the matrix.

Preservation of the antilipase activity of phenolic compounds during the contact with the gastric juice and bile was also studied. As can be seen from the data in Fig. 2, the antilipase activity of phenolic compounds decreased mainly during the contact with the gastric juice. The bile had less effect on the antilipase activity. The residual activity of the inhibitor was low (40.4 % of the initial value). These results correlated with data for the pH stability of the phenolic compounds.

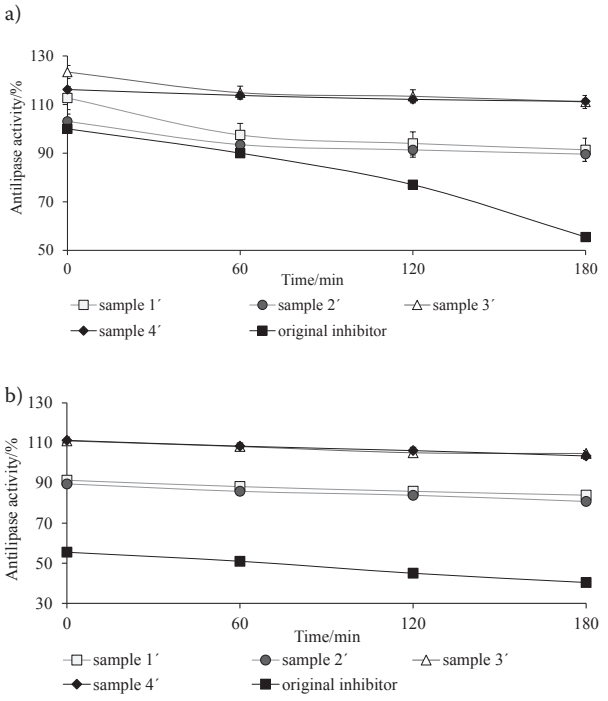

Fig. 2. Changes in the antilipase activity of samples 1'–4' under the conditions simulating normal digestion in: a) gastric juice and b) bile

We also studied the temperature effect on the antilipase activity of the inhibitor. It was important to predict the inhibitor stability in the human body when choosing the temperature conditions during preparation, drying and storing the samples of the immobilized inhibitor.

It was shown that the lipase inhibitor was stable at (20±2) °C for 120 min of incubation (Fig. 3). However, when the phenolic compounds were kept at the same temperature for a long time, their activity was significantly reduced. If the phenolic compounds extracted from the rapeseed were kept at (20±2) °C for 6 h, their antilipase activity was reduced by 26.0 % of the initial value. When incubation temperature increased, the antilipase activity of phenolic compounds decreased after 60 min. Thus, it was necessary to investigate the influence of temperature in the range from 20 to 40 °C on the immobilization of phenolic compounds on the matrix, and on drying of the immobilized inhibitor at (65±2) °C.

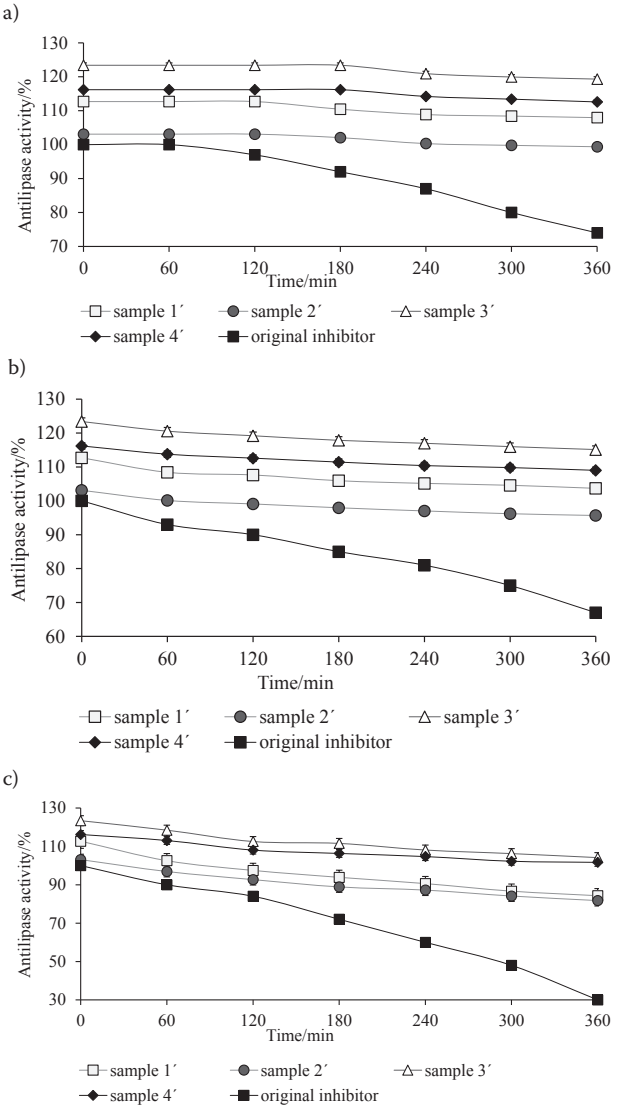

Fig. 3. Stability of samples 1'–4' at temperatures of: a) (20±2), b) (37±2) and c) (65±2) °C

When the phenolic compounds were stored at (20±2) °C and relative humidity of 75 %, their antilipase activity decreased by 32.1 % after 1 day, by 51.4 % after 3 days, by 69.1 % after 5 days and by 85.0 % of the initial value after 7 days.

Thus, phenolic compounds extracted from the rapeseed inhibited the activity of pancreatic lipase. However, their effect was significantly reduced in the acid medium at pH=2. In addition, the antilipase activity of the inhibitor significantly decreased at higher temperature, as well as during the long-term storage at (20±2) °C. This indicates out the necessity to stabilize the phenolic compounds.

Immobilization of lipase inhibitor on the matrices

Stabilization of the inhibitor was carried out by its immobilization on a matrix of *Agaricus bisporus* cell walls. Their chemical composition was shown in Table 3. They present a complex of biopolymers: polysaccharides, melanin and proteins. Samples 1–4 were obtained under different conditions and therefore the ratio of their components differed. In the polysaccharide hydrolyzate, only glucose

Table 3. The chemical composition of samples of matrices

Sample	Conditions		*w*/%			
	(*m*(NaOH)/*V*(solution))/%	Time (exposure)/min	Glucan	Chitin	Protein	Melanin
1	3	60	56.5±1.2	14.0±0.3	11.5±0.2	13.8±0.3
2		300	59.8±1.3	15.8±0.3	9.2±0.2	11.9±0.2
3	7	60	47.9±1.0	18.8±0.4	11.1±0.2	18.8±0.4
4		300	38.8±0.8	41.7±0.8	3.0±0.1	13.6±0.3

Data are expressed as mean value±S.D. of triplicate measurements

and glucosamine were detected by by gas liquid chromatography. Therefore, we concluded that polysaccharides were represented by glucan and chitin, which dominated in the samples (66.7–80.5 %). Chitin, compared to the other matrix components, is insoluble in the alkaline solutions. Therefore, the increase of the mass per volume ratio of the alkaline solution and extraction time increased the extraction of glucan and proteins. As a result, chitin content in the samples increased. This was especially clear in sample 4, which was obtained by using the alkaline solution with a maximum mass per volume ratio (7.0 %) and extraction time (300 min).

Effect of inhibitor content and chemical composition of the matrix on antilipase activity of immobilized inhibitor

The original inhibitor was stable at 20–25 °C for 60 min (Fig. 3). That is why its immobilization was carried out under these conditions.

The samples of the immobilized inhibitor 1'–4' were obtained as a result of the inhibitor interaction with matrices 1–4. The influence of the inhibitor content in the matrix on the antilipase activity of the immobilized inhibitor is shown in Table 4. When the content of the inhibitor in the sample increased, the antilipase activity of the immobilized inhibitor also increased. When the inhibitor content was 12 %, the antilipase activity of the immobilized inhibitor exceeded the activity of the original inhibitor in all samples. In samples 3' and 4' this effect was observed even if the content of the inhibitor in the sample was lower. A further increase in the inhibitor content to 16 % did not lead to a significant change in the antilipase activity of the samples. Based on the obtained data, it was concluded that samples 1'-4' with 12 % inhibitor should be used in the following experiments.

To objectively understand these results, additional studies were carried out. It was shown that matrices 1–4 had antilipase activity. In their presence, the hydrolysability of olive oil by lipase was reduced by 7.2–8.1 %. Sample 3 had the maximum antilipase activity. After the removal of melanin from matrix 3, matrix 5 was obtained and the immobilization of the inhibitor in the mass per volume ratio of 7 % was carried out on it. The antilipase activity of this sample was 1.3 times lower than that of sample 3', and it made up 80.5 % of antilipase activity of the original inhibitor.

The high antilipase activity of the immobilized inhibitors 1'–4' was probably due to the synergistic effect, which was a result of the simultaneous presence of rapeseed phenolic compounds and *Agaricus bisporus* melanin. Melanin is classified as the condensed phenolic compound, which was confirmed by the fact that sample 3, with the highest content of melanin, had the maximum antilipase activity.

Effect of immobilization conditions on antilipase activity of the immobilized inhibitor

The analysis shows that the temperature of 20–25 °C was the most favourable for immobilization, preserving the antilipase activity of the obtained samples (Fig. 4a). When the immobilization temperature increased, the antilipase activity of the immobilized inhibitor consistently decreased. This fact was observed in all used matrices. It was concluded that the increase of the immobilization temperature most probably contributes to the destruction of labile phenolic compounds (*15,17*).

Table 4. The antilipase activity of the immobilized inhibitor

w(inhibitor)/%	Antilipase activity/%			
	1'	2'	3'	4'
1	90.1±1.8	88.1±1.7	94.3±1.9	91.3±1.8
4	90.4±1.9	88.3±1.7	95.3±1.9	91.6±1.8
7	90.8±1.7	88.9±1.8	104.7±2.0	91.9±1.8
10	100.4±2.0	95.8±1.9	116.9±2.2	104.3±2.0
12	112.7±2.2	103.1±2.0	123.4±2.3	116.2±2.2
14	112.4±2.3	102.8±2.1	123.0±2.3	117.6±2.3
16	112.8±2.3	101.6±2.0	122.3±2.2	117.0±2.2

Data are expressed as mean value±S.D. of triplicate measurements

When the immobilization time was increased up to 30 min, the antilipase activity of the immobilized inhibitor also increased (Fig. 4b). During this time the phenolic compound solution probably penetrated into the matrix, which improved the efficiency of immobilization. Increasing the duration of the process by more than 30 min did not give a positive result. Thus, it was expedient to carry out immobilization at the temperature of 20–25 °C for 30 min.

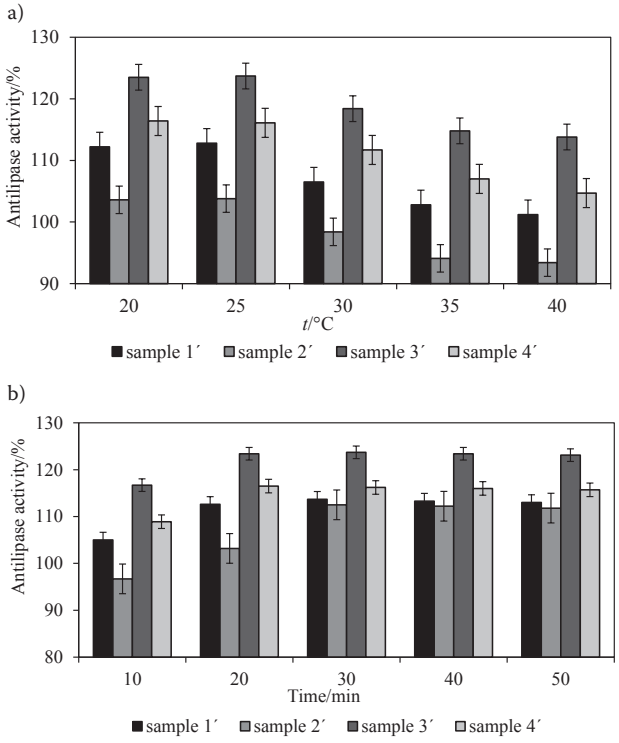

Fig. 4. The effect of immobilization conditions on the antilipase activity of samples 1'–4': a) temperature and b) time

Binding of inhibitor immobilization in the matrix

To confirm the inhibitor binding, the process of inhibitor immobilization was analyzed on sample 3. After the contact of sample 3' (containing 12 % of the inhibitor) with water at (37±2) °C during 2 h, only 4 % of the initial phenolic compounds were found in the obtained solution. The phenolic compounds that passed into the solution showed no antilipase activity. Thus, it was concluded that the matrix completely adsorbed the phenolic compounds with antilipase activity.

When comparing the IR spectra of the original inhibitor and the immobilized inhibitor (samples 1'–4' with 12 % of the inhibitor), it was found that the intense broad band with a maximum absorption at 3400 cm^{-1} was shifted to the low frequency region in comparison with the frequency of free OH groups. This indicated the presence of hydroxyl groups in hydrogen bonds. The intensity of the absorption bands at 1648–1690 cm^{-1} decreased. The absence of an absorption band of 3650 cm^{-1} led to the conclusion that practically all hydroxyl groups were part of the formed hydrogen bond.

The differential thermal analysis data are given in Table 5. At temperatures of 100 to 120 °C, the mass reduction of both the immobilized inhibitor (samples 1'–4' that contained 12 % of the inhibitor) and the mixture of the inhibitor and samples 1–4 was detected because of the removal of free moisture, followed by the endothermic effect. When the temperature reached 360 °C, the mass reduction of only the immobilized inhibitor was detected, followed by exothermic effect. This effect was not observed when the mixture of the inhibitor and samples 1–4 was used. An additional exothermic effect at 360 °C of the immobilized inhibitor was probably caused by the destruction of the inhibitor-matrix bonds, which confirmed the immobilization of the inhibitor on the matrix. At higher temperature, the abrupt decrease in the mass of both the immobilized inhibitor and the mixture of the inhibitor and samples 1–4 was detected due to their destruction. Since the samples taken for the study contain the same amount of the inhibitor, there was no significant difference in their characteristics evaluated using this method. The results of thermal analysis and IR spectroscopy confirmed that immobilization of the lipase inhibitor on the matrix took place and that the inhibitor-matrix complex was formed by hydrogen bonds.

As it is known, the immobilization methods can be divided into chemical and physical methods. Physical methods are characterized by weaker interactions such as hydrogen bonds, hydrophobic interactions, Van der Waals forces, affinity binding, or mechanical containment of biologically active substance within the matrix. In the chemical method, formation of covalent bonds is achieved through ether, thioether, amide or carbamate bonds between the biologically active substance and matrix (*36*). There are four principal techniques for immobilization of the biologically active substance, namely adsorption, entrapment, covalent bonding and cross-linking (*36*). Thus, on the basis of the obtained data, it can be concluded that the adsorption took place during immobilization of the inhibitor on the matrix.

Properties of the immobilized inhibitor

The pH stability, thermal stability and the stability of the inhibitor immobilized on biopolymer matrix during storage were evaluated. Immobilized inhibitor samples 1'–4' with 12 % of the inhibitor were used. It was found that after 360 min of incubation at pH=2, the antilipase activity of the immobilized inhibitor was more than twice higher than the activity of the original inhibitor (Fig. 1), which was also the case after incubation in the medium with a higher pH.

It was determined that samples 3' and 4' had maximum stabilizing effect in comparison with other samples of immobilized inhibitor. In the acid medium at pH=2 and alkaline medium at pH=9, their antilipase activity was 1.3 times higher than of samples 1' and 2'.

However, the pH stability data of the immobilized inhibitor were not sufficient to predict the preservation of its biological activity in the digestive tract. That is why the influence of the solutions that simulate liquid media of the gastrointestinal tract on the stability of immobilized inhibitor was studied.

The obtained results correspond to the data on the pH stability of the immobilized inhibitor samples (Fig. 2). The

Table 5. Thermal effects of samples 1'–4'

Sample	t/°C	t_{max}/°C	Thermal effect
1'	50-120	105	endo
	120-400	360	exo
	400-900	535	exo
2'	50-120	108	endo
	120-400	365	exo
	400-900	530	exo
3'	50-120	110	endo
	120-400	369	exo
	400-900	540	exo
4'	50-120	112	endo
	120-400	368	exo
	400-900	543	exo
1+inhibitor	50-120	102	endo
	120-900	520	exo
2+inhibitor	50-120	105	endo
	120-900	523	exo
3+inhibitor	50-120	101	endo
	120-900	528	exo
4+inhibitor	50-120	107	endo
	120-900	526	exo

antilipase activity of the immobilized inhibitor was 2.0–2.6 times higher than that of the original one. This fact made possible to predict preservation of high activity of the immobilized inhibitor in human digestive tract.

The significant increase in the stability of phenolic compounds as a result of immobilization was probably due to the fact that matrices 1–4 contained chitin. This polysaccharide consists of N-acetyl-D-glucosamine units. During alkaline treatment of the samples at high temperature, deacetylation took place and amino groups are released (*19*). As a result of this process, the matrix shows antacid properties, which are the result of the substance ability to increase the pH value of acid medium. Matrices 3 and 4 were obtained by long-term alkaline treatment (300 min) and, as it was shown in a previous study (*21*), these matrices contained more amino groups than matrices 1 and 2. As a result, matrices 3 and 4 had more pronounced antacid properties than matrices 1 and 2, and they better preserve the properties of phenolic compounds in acid medium.

Thermal stability of the inhibitor also increased as a result of its immobilization (Fig. 3). High antilipase activity of samples 1'–4' at (37±2) °C for 6 h was observed. That is why at the temperature of the human body they show the antilipase activity. After incubation of the original inhibitor at (65±2) °C for 360 min, its residual antilipase activity was 30 %, which was 2.7–3.5 times lower than the residual activity of the immobilized inhibitor. The samples 1'–4' were stable at (65±2) °C. That is why we recommend them for dry use by both freeze drying and spray drying. The thermal and the pH stability of samples 3' and 4' were higher than of samples 1' and 2'.

Another important characteristic of the immobilized inhibitor was the preservation of its antilipase activity during storage. After 6 months of storage, the antilipase activity of samples 1'–4' with 12 % of the inhibitor was 88.5–112.8 % of the activity of the original inhibitor, but the original inhibitor lost 85.0 % of its activity after 7 days of storage (Fig. 5). After 6 months of storage, samples 2' and 4' had the highest antilipase activity. After 12 months of storage, the antilipase activity of samples 1'–4' remained at a high level, but of sample 4' was significantly higher than of other samples.

The stability of samples of the immobilized inhibitor with the increase of temperature to (37±2) and (65±2) °C, as well as during long-term storage at (20±2) °C was stipulated by the fact that the matrix prevented the transformation of rapeseed phenolic compounds. As it is known, the change in the phenolic compounds structure led to the loss of their properties. As a result, the phenolic compounds did not show any antilipase activity. Such transformation of their properties could be the result of the oxidation due to contact with oxygen from the air (*17*).

As it is shown in a previous study (*21*), matrices 1–4 had high antioxidant properties. Such properties can be explained by the presence of melanin in their composition and free amino groups in the chitin molecule. Therefore, they were able to inhibit the oxidation of rapeseed phenolic compounds in contact with oxygen from the air and preserve their antilipase activity.

Analyzing the obtained results, it should be noted that high pH stability, thermal stability and stability of the immobilized inhibitor during storage were related to the prop-

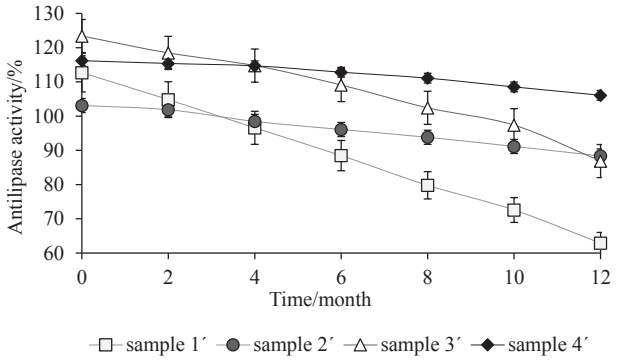

Fig. 5. Changes in the antilipase activity of samples 1'–4' during storage

erties of matrix biopolymers. Immobilization of biologically active substances usually led to the partial decrease in their activity (*19,20,36,37*). For example, after the immobilization of protease on polysaccharides isolated from cereals, the activity of the immobilized enzyme was 55 % of the activity of the original enzyme (*37*). Lipase immobilized on cereal polysaccharides showed 72.4 % of the initial enzyme activity (*37*). After the protease inhibitor was immobilized on cereal polysaccharides, the obtained immobilized inhibitor had only 33 % of the activity of the original inhibitor. That is why in this work the matrices with antilipase activity where chosen, and during immobilization the synergistic effect was observed, so as a result, the activity of phenolic compounds did not decrease and in samples 1'–4' it exceeded the activity of the original inhibitor.

As it is known, immobilization of the biologically active substances leads to the increase of their resistance to changes in environmental conditions (*19,20,35,36*). For example, after incubation of protease, lipase and protease inhibitor in acid medium, they completely lose their activity. However, all immobilized samples had higher pH stability than the original biologically active substances. After their incubation in the acid medium, the activity of the immobilized biologically active substance was around 20–45 % of the activity of the original biologically active substances.

Due to the fact that the matrix also contributed to the preservation of the activity under different pH and temperature, in this work the matrices that could preserve the antilipase activity of the phenolic compounds were chosen. As a result of the immobilization of rapeseed phenolic compounds on the matrix, the pH stability, thermal stability, and stability during storage of the immobilized inhibitor were enhanced. Apart from that, the antilipase activity of the immobilized inhibitor after its incubation in the acid medium was notably higher than the activity of the above-mentioned biologically active substance, due to the fact that the used matrices had antacid and antioxidant properties. Thus, the immobilization of the inhibitor on biopolymers from *Agaricus bisporus* provided the stable form of the lipase inhibitor.

In most cases, during changes in the environmental conditions, the antilipase activity of samples 3' and 4' exceeded the activity of the original inhibitor. Only after storage for 12 months the activity of sample 3' insignificantly decreased. Taking into account the possibility of practical use of the obtained results for the development of production technology, it is economically viable to obtain sample 3', since the use of longer treatment at high temperature will lead to the increase of the obtained product cost.

Conclusions

The stabilized form of lipase inhibitor was obtained by its immobilization on the biopolymers from *Agaricus bisporus* cell walls. It had significantly better pH and thermal stability than the original inhibitor. It was stable under the conditions of the digestive tract. The immobilized inhibitor retained the activity for a long time during storage. The sample that had 12 % of the inhibitor and the matrix containing 47.9 % glucan, 18.8 % chitin, 18.8 % melanin and 11.1 % proteins showed the best characteristics. The best immobilization was carried out at 20–25 °C for 30 min. The hydrogen bonds between the inhibitor and the matrix were formed during immobilization.

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