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Study of the Regularities of Inhibition by Organic Acids of the Enzyme Complex Wheat Germ

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***Abstract*—Wheat germ - a product of deep processing of wheat grain is a native plant component with high nutritional and biological value. Due to the valuable biochemical composition and high functional and technological properties of the wheat germ, it has significant prospects for use in food, confectionery, perfumery industry and medicine. It has been established that ascorbic, succinic and fumaric acids effectively reduce the activity of enzymes that determine the storage capacity of wheat germ. It has been proven that compositional mixtures of succinic, ascorbic, and fumaric acids suppress the activity of the enzymes of the embryo in a non-competitive type of inhibition. The important problem of increasing the shelf life of a valuable by-product of domestic production has been solved, while respecting the concept of resource saving.**

Keywords—wheat germ, lipase, lipoxygenase, catalase, inhibition, organic acids

I. INTRODUCTION

The state program for the development of agriculture and the regulation of agricultural products, raw materials and food markets are aimed at developing the production of food products enriched with indispensable components, functional products, the dissemination of technologies for the deep processing of agricultural raw materials on the basis of non-waste production, the rational use of secondary products, and the reduction of food industry waste. From this point of view, a great interest is in the product of deep processing of wheat grain - wheat germ, which is a native plant component with high nutritional and biological value. It contains vitamins A and E, B vitamins, more than 20 macro- and microelements. Due to its valuable biochemical composition and high functional and technological properties, wheat germ has significant prospects for use in food, confectionery, perfumery and medicine. However, due to its high lipid content (8–10%), wheat germ, which contains up to 80% of polyunsaturated fatty acids (including ω-3 and ω-6 fatty acids), has low storage stability [1–14].

The root cause of rapid spoilage of wheat germ is the action of enzymes. The initiator of the process of rancidity of fat of wheat germ is lipase (EC 3.1.1.3), under the action of which the lipids are hydrolyzed with the formation of free fatty acids and further intensive oxidation of the latter. As a result, intense microbiological processes, an unpleasant odor and a sharp rancid taste in the product arise [5, 15-24].

We hypothesized that the expected antioxidant effect when using organic acids and their compositions as inhibitors of enzymatic oxidative processes also allows the formation of additional parapharmaceutical properties of wheat germ. Parapharmaceuticals - biologically active food additives, have long been used for prevention, adjuvant therapy and support in the physiological framework of the functional activity of organs and systems. For use in the food industry, preference was given to succinic, fumaric and ascorbic acids that SanPin approved for use in the food industry, which has high antioxidant activity and manufacturability, a known functional effect on the human body. These organic acids can be attributed to high-tech substances, they are loose, finely divided, homogeneous, powdery products of white color. These acids are slightly hygroscopic, do not coalesce, do not hang in operational containers, are uniformly dosed. These properties of organic acids characterize them as technological, useful nutritional supplements that help restore the functioning of organs and systems of the body, accelerate recovery and maintain the natural balance of its life. Considering the high antioxidant activity of these organic acids, availability (their industrial production has been mastered in Russia), manufacturability, sufficient cheapness, we have chosen these compounds [25-30].

The aim of the work is to study the patterns of inhibition of wheat germ enzyme complex by organic acids and their compositions.

II. EXPERIMENTAL

In the work we used wheat germ, produced industrially (TU 9295-010-00932732-08 "Wheat germinal food flakes"). In the studies, batches of wheat germ obtained from the processing of wheat grains of various varieties and types

delivered from Belgorod, Lipetsk and Voronezh regions were used.

A wheat germ lipoxygenase preparation was obtained by mechanical grinding in a laboratory mill and homogenizing the object with distilled water. Wheat germ extract was precipitated with ethanol at a temperature of 2-4 °C. The mixture was infused for 15-20 minutes, the precipitate was isolated in a centrifuge and dried under vacuum. The pH was adjusted with HCl or NaOH solutions. The enzyme precipitated within 65-85% saturation of $(NH_4)_2SO_4$. During the purification of the enzyme, gel filtration on Sephadex G-25 and G-100 (Pharmacia, Sweden) was used. During the isolation and purification of lipase, the preliminary preparation of biological material consisted in grinding wheat germ and delipidation with acetone cooled to (-10 °C). The obtained acetone powder was used for extraction of the enzyme. Subsequent research steps were carried out at a temperature of 4 °C. Acetone powder was ground in a mortar. The homogenate was centrifuged using a TsLR-1 centrifuge (Russia).

Protein precipitation of the obtained supernatant was carried out using a Magnetic stirrer Type mm6 mixer (Poland) using a Mettler brand MP 220 pH meter (Germany) by adding cold acetic acid. The mixture was infused for 10-15 minutes to form a precipitate and then centrifuged. After deposition was completed, the enzyme activity and protein content (according to Lowry) were determined in the samples. For enzyme purification, ion exchange chromatography on DEAE cellulose from Reanal (Hungary) was used. Then it was kept for 1 h in NaOH solution, then in HCl solution and again in NaOH solution. Elution with DEAE-cellulose was performed using a stepwise gradient KCl in Tris-HCl buffer, pH = 8.0. We used gel filtration on Sephadex G-25 and gel chromatography on G-150 from Pharmacia (Sweden). The homogenate was centrifuged in a refrigerated centrifuge. The enzyme was precipitated from wheat germ homogenate with ethyl alcohol at a temperature of 2-4 °C. The mixture was infused for 15-20 minutes to form a precipitate.

To find the activity of catalase, a reaction mixture consisting of an enzyme, phosphate buffer (pH = 7.4) and hydrogen peroxide was incubated at a temperature of 37 °C. In a control experiment, a solution of sulfuric acid was added to the enzyme to inactivate the enzyme, phosphate buffer (pH = 7.4) and hydrogen peroxide. The remainder of hydrogen peroxide was titrated with a solution of potassium permanganate (in the experimental and control flasks). In the control experiment, distilled water is used instead of the enzyme solution. Lipase activity was determined using the pH-staining method. The reaction mixture was heated to 37 °C and the pH was adjusted by adding sodium hydroxide solution. Next, an enzyme solution was added. The control was carried out similarly.

When studying the effect of compositions of organic acids A-D on lipoxygenase in a reaction medium containing a preparation of lipoxygenase, 0.15 mol / dm^3 of phosphate-citrate buffer (pH 7.0), alternating mixtures of acids A-D were applied to final concentrations of 8, 20, 30 mM / dm^3. Next, a substrate was introduced - refined sunflower oil. The enzyme activity was determined by changing the substrate concentration in the range of 20-100 mM / dm^3, taking the molecular weight of the oil 913 Da (with constant monitoring of pH in the reaction media). The mixture was incubated with constant stirring for 20 min at a temperature of 30 °C and the residual enzyme activity was determined.

Crystalline ascorbic, succinic and fumaric acids in quantities of 1-7% were mixed with wheat germ for 4 min in a laboratory mixer and stored in a refrigerator (temperature 4-6 °C, relative humidity 75-80%).

III. RESULTS AND DISCUSSION

The most pronounced positive effect was noted starting with an acid concentration of 5% or more, the results of experimental studies on changes in the activity of wheat germ enzymes (at a concentration of organic acid in a mixture of 5%) are presented in Table I. In the control sample untreated with organic acids is marked intensive growth in the activity of enzymes that regulate the processes of lipid peroxidation of wheat germ. The research results indicate that ascorbic, fumaric and succinic acids have antimicrobial effects on wheat germ and inhibitory properties against enzymes of wheat germ to varying degrees (Table II).

TABLE I. CHANGES IN THE ACTIVITY OF LIPASE, LIPOXYGENASE AND CATALASE OF WHEAT GERMIN THE PRESENCE OF ORGANIC ACIDS

Quality indicators	**Product option**	**Storage time, weeks**				
		Start	***2***	***4***	***6***	***8***
Lipase activity, μm / min · mg	Control	3,9465	4,2369	4,9271	5,4298	6,1037
	Ascorbic acid		3,4925	3,0195	2,7449	2,5987
	Fumaric acid		3,7254	3,5922	3,4120	3,3125
	Succinic acid		3,6244	3,4983	3,4027	3,3925
Lipoxygenase activity, μm /ml·min	Control	4,1949	4,5036	5,0372	5,7935	6,8712
	Ascorbic acid		3,6884	3,2910	3,0889	2,9601
	Fumaric acid		3,8973	3,8214	3,7986	3,7089
	Succinic acid		4,0891	3,9732	3,9032	3,8601
Catalase activity, μm /mg·min	Control	3,4750	4,2001	4,9633	5,5771	5,9102
	Ascorbic acid		3,0962	2,7112	2,5077	2,4300
	Fumaric acid		3,2945	3,1027	3,0698	2,9603
	Succinic acid		3,3965	3,2096	3,1548	3,1346

TABLE II. THE QUALITY OF WHEAT GERM AFTER 2 MONTHS OF STORAGE IN THE PRESENCE OF ORGANIC ACIDS

Quality indicators	Ascorbic acid	Fumaric acid	Succinic acid
Lipase activity, μm / min · mg	2,5987	3,3125	3,3925
Lipoxygenase activity, μm /ml·min	2,9601	3,7089	3,8601
Catalase activity, μm /mg·min	2,4300	2,9603	3,1346
Peroxide value, mm / kg	11,23	12,26	13,63
Total seeding, CFU / g	$4{,}0 \cdot 10^4$	$3{,}9 \cdot 10^4$	$4{,}2 \cdot 10^4$

The results of experimental studies of qualitative indicators of wheat germ with an organic acid content of 5% after 2 months of storage show that ascorbic acid has the most pronounced inhibitory effect on lipase, lipoxygenase and catalase of wheat germ (Table II). Fumaric acid had the strongest antimicrobial effect on the stored product; the seeding value in the test sample after 2 months of storage was $3.9 \cdot 10^4$ CFU / g, which is $0.1\text{-}0.3 \cdot 10^4$ CFU / g less than in the samples treated with ascorbic and succinic acids.

The data obtained make it possible to predict the properties of composite mixtures of the studied acids for the purpose of their subsequent targeted use for stabilization and enrichment of wheat germ. For this purpose, the following compositions of organic acids were investigated: A - ascorbic and fumaric acids (1: 1); B - ascorbic and succinic acids (1: 1); C - succinic and fumaric acids (1: 1); D - ascorbic, succinic and fumaric acids (1: 1: 1). Prototypes were processed and stored according to the methodology above. It was experimentally proved that organic acid compositions have a more significant inhibitory effect compared to individual acids: lipase activity decreased by 10%, lipoxygenase - by 9% and catalase - by 15%, respectively. Further studies were aimed at studying the inhibitory properties of organic acid compositions.

Fig. 1 shows the results of studies of the dependence of the rate of the enzymatic reaction of wheat germ lipoxygenase on the substrate content in media in the presence of organic acid compositions.

From Fig. 1 it follows that mixtures of organic acids intensively reduce the rate of the enzymatic reaction of lipoxygenase. The higher their concentration, the greater the inhibitory effect. Applying the Linewiver – Burke method [31-33] and using the information presented in Fig. 1, we analyzed the functions 1/v and 1/[S]. The results are shown in Fig. 2.

Fig. 2 shows that all graphs can have a common point on the abscissa axis when interpolating the experimental dependences in the region of negative values. This information shows that the compositions of acids A-D actively reduce the rate of the enzymatic reaction of lipoxygenase in a non-competitive manner. The results of the calculation of Michaelis constants are shown in Table III. Km characterizes the chemical similarity of the enzyme to the substrate, the higher it is, the lower the rate of the enzymatic reaction v and the greater the effect of the inhibitory effect.

TABLE III. MICHAELIS CONSTANT VALUES FOR LIPOXYGENASE WHEAT GERM IN THE PRESENCE OF MIXTURES A-D

Composition name	Component name	Michaelis constant, Km
Mixture A	Ascorbic and fumaric Acids	66,7
Mixture B	Ascorbic and succinic acids	200,0
Mixture C	Succinic and fumaric Acids	142,9
Mixture D	Ascorbic, succinic and fumaric acids	250,0

From the data obtained, it can be concluded that the effect of the inhibitory effect of the composite mixtures A, C on the lipoxygenase of wheat germ was the smallest, the composition of acids B and D had a more significant inhibitory effect.

Fig. 3 shows the dependences of the rate of the enzymatic reaction of lipase on the amount of substrate in reaction media containing different concentrations of organic acids A-D. Fig. 3 shows that AD mixtures inhibit the reaction rate symbatically. To identify the type of inhibition, 1/v and 1/[S] functions were analyzed, the results are shown in Fig. 4.

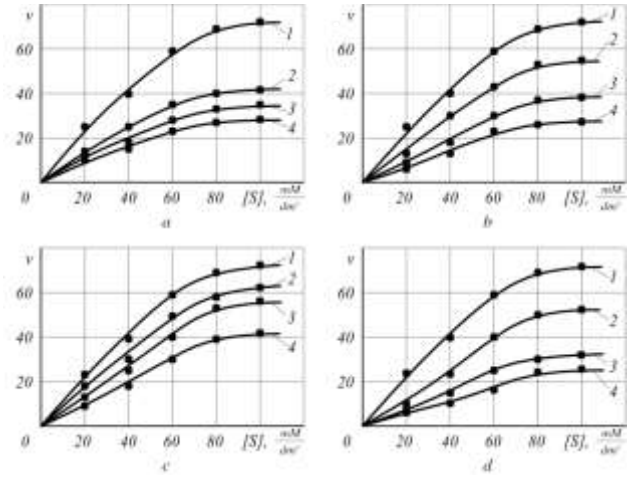

Fig. 1. The effect of substrate concentration ([S], mM /dm^3) on the rate of the enzymatic reaction of wheat germ lipoxygenase (v, units) in the presence of mixtures A (a), B (b), C (c), D (d), mm / dm^3:1 - control; 2 - 8; 3 - 20; 4 - 30.

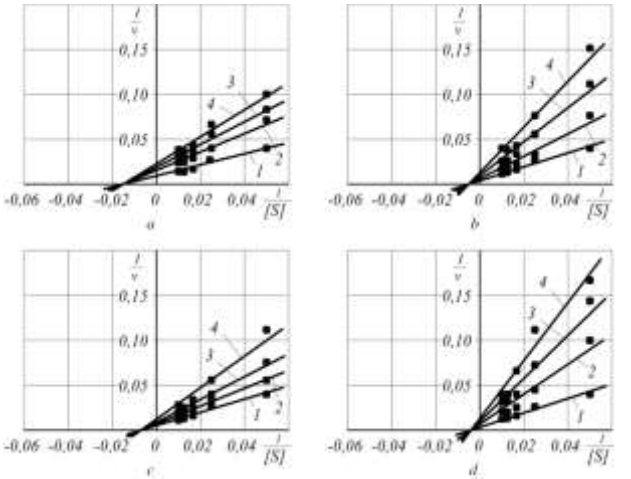

Fig. 2. Dependence 1/v = f (1/[S]) for wheat germ lipoxygenase in the presence of mixtures A (a), B (b), C (c), D (d), mM/dm^3: 1 - control; 2 - 8; 3 - 20; 4 - 30.

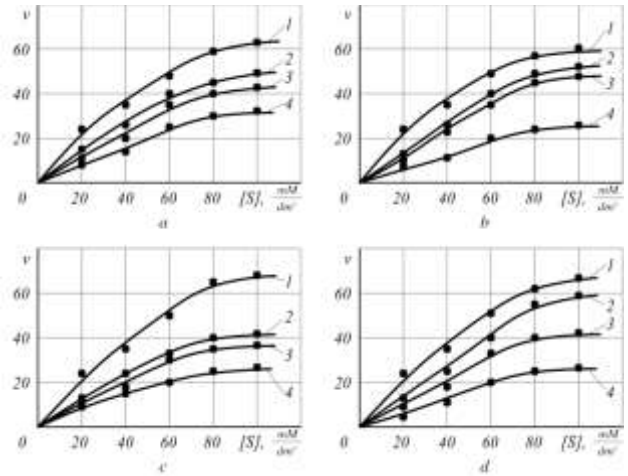

Fig. 3. The effect of substrate concentration ([S], mM / dm^3) on the rate of enzymatic reaction of wheat germ lipase (v, units) in the presence of mixtures A (a), B (b), C (c), D (d), mM / dm^3: 1 - control; 2 - 8; 3 - 20; 4 - 30.

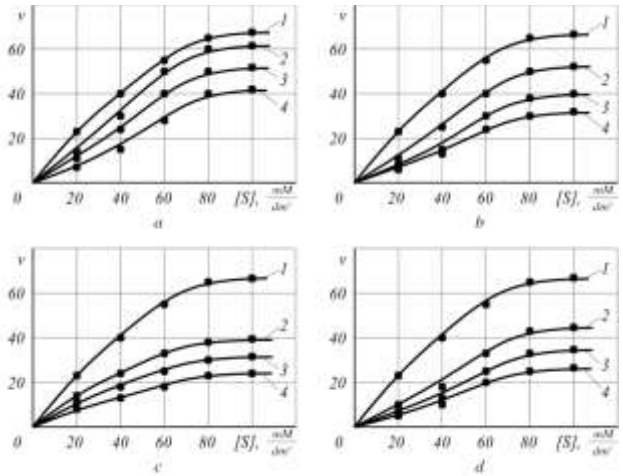

Fig. 5. The effect of substrate concentration ([S], mM/dm^3) on the rate of enzymatic reaction of catalase wheat germ (v, units) in the presence of mixtures A (a), B (b), C (c), D (d), mm/dm^3: 1 - control; 2 - 8; 3 - 20; 4 - 30.

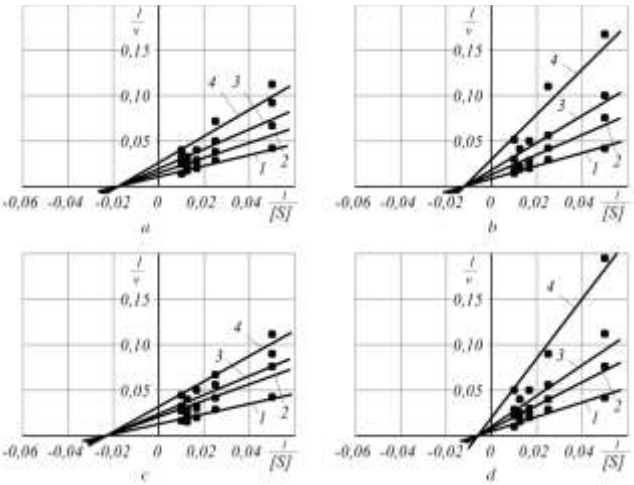

Fig. 4. Dependence 1/v = f (1/[S]) for wheat germ lipoxygenase in the presence of mixtures A (a), B (b), C (c), D (d), mM / dm^3: 1 - control; 2 - 8; 3 - 20; 4 - 30.

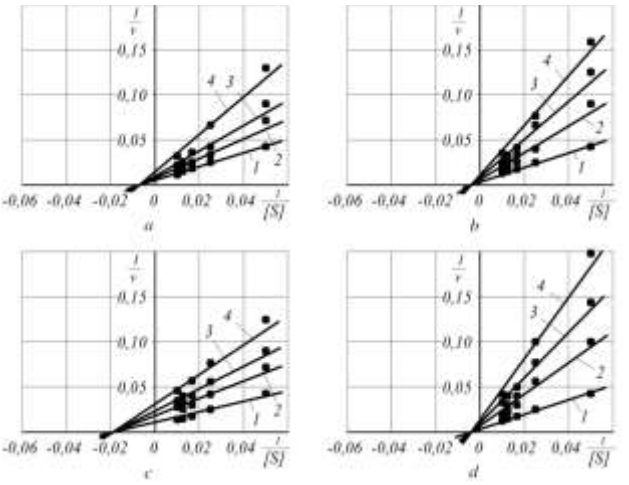

Fig. 6. Dependence 1/v = f (1/[S]) for wheat germ catalase in the presence of mixtures A (a), B (b), C (c), D (d), mM/dm^3: 1 - control; 2 - 8; 3 - 20; 4 - 30.

From Fig. 4 it follows that all graphs can have a common point on the abscissa axis when interpolating experimental dependences in the region of negative values, and the slope of the graphs to the abscissa axis increases with increasing concentration of mixtures of organic acids, which indicates a non-competitive type of inhibition. Calculation of Michaelis constants showed that in the presence of mixtures A, B, C, D, they have the following values, respectively: 55.6; 83.3; 43.5; 166.7. The data obtained show that the greatest inhibitory effect on the lipase of wheat germ was exerted by compositions B, D, the least - A, C.

Fig. 5 shows the dependence of catalase activity on the amount of substrate in media containing AD compositions. Using the Linuiver-Burke method, applying the results obtained, we plotted the dependences 1/v = f (1/[S]), presented in Fig. 6. From Fig. 6 it follows that all graphs can have a common point on the abscissa axis when interpolating experimental dependencies in the region of negative values. The data obtained show that acid compositions intensively reduce the rate of enzymatic catalase reaction of wheat germ in a non-competitive manner.

We calculated the Michaelis Km constants for catalase of wheat germ in the presence of mixtures of organic acids AD (Table IV). From the data of Table IV it can be seen that a stronger inhibitory effect on the catalase of wheat germ had a composition of organic acids B, D, less - a mixture of A, C.

TABLE IV. MICHAELIS CONSTANT FOR CATALASE WHEAT GERM IN THE PRESENCE OF MIXTURES A-D

Composition name	**Component name**	**Michaelis constant, Km**
Mixture A	Ascorbic and fumaric Acids	138,9
Mixture B	Ascorbic and succinic acids	238,1
Mixture C	Succinic and fumaric Acids	83,3
Mixture D	Ascorbic, succinic and fumaric acids	256,4

IV. CONCLUSION

It has been proven that ascorbic, succinic and fumaric acids effectively reduce the activity of enzymes that determine the storage capacity of wheat germ. It was found that composite mixtures of succinic, ascorbic and fumaric acids inhibit the activity of wheat germ enzymes in a non-

competitive type of inhibition. The synergistic effect of the compositions of organic acids was revealed in comparison with the individual components. The introduction of composite mixtures of acids in an amount up to 3-5% by weight of wheat germ reduced the activity of enzymes by 10-15%, with 5-7% - by 70-80%. Thus, the important problem of increasing the shelf life of a valuable by-product of domestic production, while respecting the concept of resource conservation, is solved. The obtained research results can be applied in the development of parameters and storage modes of wheat germ, with additional enrichment of a new type of raw material with parapharmaceuticals.

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