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# THE EFFECT OF THE PROTEIN-FAT PREPARATIONS AUTOOXIDATION IN THEIR ENZYMATIC DIGESTIBILITY AND SOLUBILITY

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Key words: protein digestibility, fat digestibility, peroxide number.

Changes in fat and protein digestibility as well as in the solubility of high-caloric protein-lipid preparations during storage at accelerated autoxidation were investigated. It was observed that fat and protein digestibility gradually decreased and the solubility of preparations was considerably reduced with the growth of the peroxide number.

Non-oxidised fats do not react with proteins, and if they do, they produce non-lasting bonds. On the other hand, the effect of oxidised fats on protein may result in many undesirable changes both in the course of food production and storage. These interactions often lead to the deterioration of the nutritive value of products expressed i.a. with the decrease of the content of such essential aminoacids as triptophane and available lysine [7]. Another effect may also be the poorer protein and fat digestibility degree and their suceptibility to the action of digestive enzymes. The investigations carried out by Pokorny [3, 4], Andrews [1] and Tappe [8] indicate that the protein-oxidated-fat complexes decompose during alkaline and acid hydrolysis, but under the effect of proteolytic enzymes they decay only partially this being ascribed to the development of cross linkings between oxidated fats and proteins. However the experimental models assumed by various researchers, the conditions under which the protein-fat links were being obtained as well as methods used to evaluate the changes taking place, greatly differ

from one another thus making it difficult to compare the results obtained and to carry out some synthetical analysis.

The purpose of this study was to determine the changes in the degree of fat and protein susceptibility to enzymatic digestion and the solubility of the high-alorific protein-fat preparations during autooxidation.

## MATERIAL AND METHODS

Protein-fat preparations obtained from:

— fresh skimmed milk (acidity 6.7°SH), pasteurised and afterwards concentrated in an Alfa-Laval evaporator, type CT – 1B to 40% of dry substance,

— refined soya bean oil (Lee number 1.28, iodine number 119.2)

were used for testing.

These two ingredients, at a ratio of 1:1 (in terms of dry substance) were homogenized at 60°C under a pressure of 70 atm (preparation "a") and 200 atm (preparation "b"). The remaining parameters were the same as quoted in the previous study [7].

The conditions of autooxidation of preparations being tested, the methods of determination of free fat, total fat, water and total nitrogen, as well as the way in which the bounded and free fat was extracted for the determination of the peroxide and iodine numbers, were described in detail in the former publication [7]. The determination of amine nitrogen, the digestibility of protein anf fat and the solubility of preparations under tests were carried out as described below.

### A. DETERMINATION OF AMINO NITROGEN AND PROTEIN DIGESTIBILITY

The "in vitro" determination of protein digestibility [2, 5], i.e. digestion with proteolytic enzymes was carried out according to the Akeson and Stahman method modifying it, however, due to the specific features of the material under test. The simultaneous high content of fat and protein was a factor which hampered normal analytical operations.

Protein digestibility was determined for the two preparations and for powdered, skimmed milk (1% content of fat in terms of dry substance) which constituted the initial protein component in these preparations. This value was determined as the growth of the content of amino nitrogen after digestion:

— with pepsine,

— with pepsine and tripsine,

— with pepsine and pancreatine.

*1. Preparation of samples for digestion*

After preliminary determination of the content of total nitrogen in material under test, a water suspension was made of this material the concentration being such as to have 100 mg of raw protein in 3 ml of suspension.

*2. Determination of amino nitrogen*

Amino nitrogen was determined by the Spies-Chambers method [6], reading its content from a standard curve prepared for LD-$\alpha$-alanine. The results were given in N-amine mg in terms of 100 mg of N-total, taking into account the weight and the successive dilution of the sample.

*3. Determination of amino nitrogen prior to digestion*

7 ml of borate buffer of pH 7.4 were added to 3 ml of product tested, and shaked for 30 minutes. Then, 5 ml of 10% TCA were added and the solution was incubated for 1 hour at 37°C. After the elapse of this time the volume was filled up to 25 ml and 2.5 ml were taken from the filtrate for N-amino determination.

*4. Enzymatic hydrolysis*

5 mg pepsine dissolved in 8 ml 0.1n HCl were added to calibrated test-tubes each containing 3 ml of solution under test. The tubes were then placed in a thermostat with a water jacket of a temperature of 37°C for a period of 3 hours. Then the samples were neutralised by adding 2.0 ml of 0.4n NaOH. 10 mg tripsine dissolved in 5 ml of borate buffer of pH 7.4, or 20 mg pancreatine dissolved in 5 ml of borate buffer of pH 7.4, were added to each test-tube. Hydrolysis was carried out at 37°C for 24 hours.

**COMMENTS**

a) In order to determine amino nitrogen after digestion with pepsine, the neutralised samples were filled up to a volume of 25 ml. 10 ml were taken from each sample and to this volume 5 ml of 10% TCA were added. The samples were incubated for 1 hour at a temperature of 37°C, and filtered. 5 ml were taken from the filtrate, 2.5 ml of 0.4n NaOH were added and the content was topped up to a volume of 10 ml; 2.5 ml were taken from each 10 ml sample for amino nitrogen determination.

b) In order to determine amino nitrogen content after digestion with pepsine and tripsine or pancreatine, the hydrolysates were filled up to a volume of 25 ml. 10 ml were take from the test-tubes and 5 ml of 10% TCA were added. The whole was incubated for 1 hour at 37°C, and then filtered. To the 5 ml taken from the filtrate, 2.5 ml of 0.4n NaOH were added and the content was filled up to a volume of 25 ml. From this solution 2.5 ml samples were taken for amino nitrogen determination.

## B. DETERMINATION OF FAT DIGESTIBILITY

The determination of fat degestibility was carried out according to a method quoted by Tysarowski [9] with some modifications necessary due to the nature of product under test. The lipolytic digestibility degree was characterised using the following enzymes:

— lipase,

— lipase after preliminary digestion with pepsine,

— pancreatine after preliminary digestion with pepsine (the amount of pancreatine used was selected so as to make the activity represented by lipase contained in pancreatine equal to the activity of lipase used individually in the first two variants of the experiment).

Adopting the above mentioned model of experimentation it was assumed that like in the normal physiological conditions "in vivo", while digesting the protein component of the envelope the proteolytic enzymes, simultaneously increased the accessibility of the fat component to pancreatic lipase.

### *1. Digestion with lipase*

10 mg of lipase dissolved in 1 ml of phosphatic buffer of pH of 7.4, 1 ml of distilled water, 100 mg of sample dissolved in 1.0 ml distilled water, 3 ml of phosphate buffer of pH 7.4 and 3 ml of 2% solution of $CaCl_2$ were added, shaking continuously, to an Erlenmeyer flask. After careful mixing the flask was placed in a thermostat at a temperature of 37°C for a period of 3 hours. Then, 10 ml of 96% ethyl alcohol was added (to break enzymatic reaction) and a few drops of 0.1% phenyphthaleine, and the content was titrated with 0.01 soda lye until it acquired red colour. Simultaneously a control sample containing identical components except lipase was made (4 ml phosphate buffer were added instead of 3 ml in the sample under test). The triglycerides hydrolysis degree was expressed as a difference in mln of 0.01n NaOH used to neutralise fatty acids in the test and control samples.

*2. Digestion with pepsine*

100 mg of sample dissolved in 1.5 ml distilled water and 5 mg pepsine dissloved in 1.5 ml of 0.2 n HCl were poured into an Erlenmeyer flask. After careful mixing the flask was placed in a thermostat at a temperature of 37°C for 3 hours. Then the sample was neutralised with 0.4 n NaOH and 10 mg lipase dissolved in 1 ml phosphate buffer of pH 7.4, 3 ml phosphate buffer of pH 7.4 and 3 ml of 2% solution of $CaCL_2$, were added to it. Further procedure was as in lipase digestion. The control sample did not contain pepsine or lipase (1.5 ml of 0.2n HCl were added instead of pepsine, and 4 ml of phosphate buffer instead of the 3 ml in the sample tested).

*3. Digestion with pepsine and pancreatine*

The procedure was the same as in pepsine and lipase digestion, but lipase was replaced by 20 mg of pancreatine dissolved in 1 ml of phosphate buffer of pH 7.4.

C. DETERMINATION OF SOLUBILITY OF PREPARATION

The solubility of the milk powder and the preparations being tested was determined according to the Polish Standard PN-65/A-86030.

## RESULTS AND DISCUSSION

In this study two preparations differring only by the homogenisation pressure applied in the production process, were used as the material for testing. The difference (70 atm and 200 atm) of this parameter was introduced to simultaneously catch the dependence of these discriminants on the fat dispersion degree. The homogenisation pressure of 70 atm enabled a preparation of the same composition of initial components to be obtained as that obtained at a pressure of 200 atm., but with an increased content of free fat which is decisive for the oxidation process. Investigations embraced only the autooxidation process induction period, in order to catch the changes occurring at a relatively small advancement of reactions caused by the action of oxygen, as is the case in the storage, in practice, of products maintaining their full consumptive value. Hence, the dependences observed show a slightly different character than those described in the previous publication [7].

As is shown in Table, the content of free fat and the fat bound in the induction period is basically maintained on an unchanged level which indicates that the protein fat interactions in this period are rather

small. Changes of numbers characteristic of the oxidation process in free fat and fat bound in the induction period, are shown in Fig. 1. These changes are more essential for free fat. Changes of the iodine and peroxide numbers in the bound fat reveal the decidedly protective effect of protein. At the same time, changes in the degree of lipolytic digestibility of preparations tested (Fig. 2) have been determined. Comparing the results obtained for pure soya bean oil and protein preparations with this oil added, one can state that the latter feature a decidedly better digestibility of the fatty component. Thus, the digestibility of the soya oil in case of digestion with lipase alone, in terms of millilitres of 0.01n NaOH under experimental conditions, is expressed by the

Table. Changes of the lipid fraction in preparation "a" during induction period of autooxidation

| Time of autooxidation (days) | Dry matter % | Free fat | | | Bound fat | | |
|---|---|---|---|---|---|---|---|
| | | in % of total fat | Lea number | iodine number | in % of total fat | Lea number | iodine number |
| 0 | 97.1 | 72.7 | 1.7 | 117.6 | 27.3 | 1.6 | 115.6 |
| 5 | 97.8 | 73.1 | 1.6 | 103.1 | 26.9 | 1.6 | 160.2 |
| 10 | 98.1 | 73.2 | 3.0 | 98.5 | 26.8 | 2.8 | 96.3 |
| 15 | 98.1 | 72.9 | 9.2 | 97.8 | 28.1 | 7.2 | 93.8 |
| 20 | 98.5 | 73.0 | 10.0 | 97.1 | 27.0 | 7.2 | 90.7 |
| 25 | 99.3 | 73.2 | 45.20 | 95.7 | 26.8 | 7.8 | 84.5 |

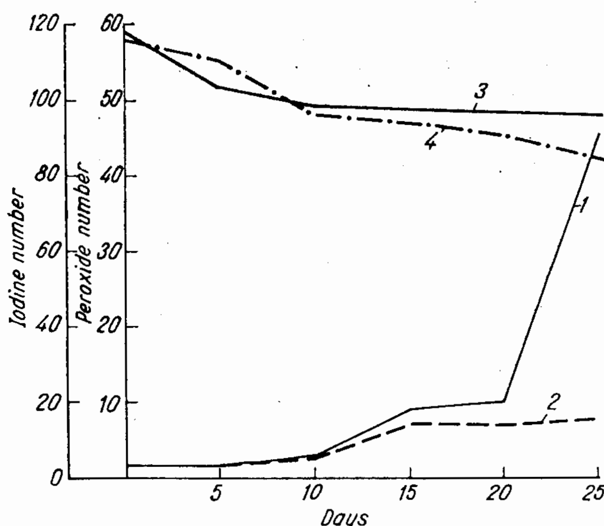


Fig. 1. Changes of Lea number and iodine number in extracted free and bound fat; 1 — Lea number in free fat, 2 — Lea number in bound fat, 3 — iodine number in free fat, 4 — iodine number in bound fat

number 6.60, and for preparations "a" and "b" 15.5 and 17.6, respectively.

If the preparations are preliminarily digested with pepsine and, then, with lipase, the digestibility of fat contained in them grows markedly to be 21.8 and 18.8, respectively; and in the case of successive use of pepsine and the pancreatic enzymes complex — 21.9 and 19.6. Thus it can be assumed that the increase in digestibility of fat in preparation undet test, after threating it preliminarily with protellytic enzymes, takes place mainly due to the action of pepsine, the effect of pancreatine being practically insignificant.

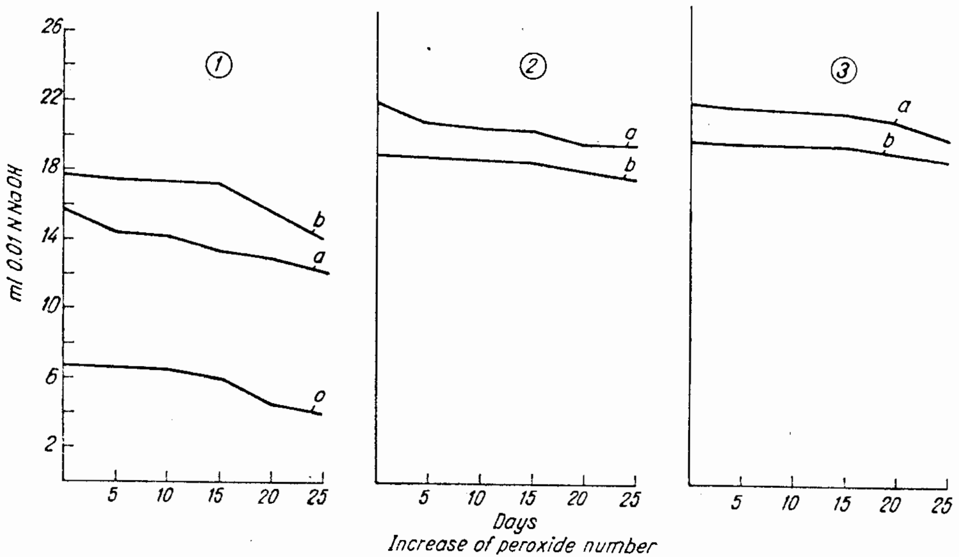


Fig. 2. Changes of lipids digestibility; o — soybean oil, a — preparation „a" (70 at), b — preparation „b" (200 at); 1 — lipase, 2 — pepsine+lipase, 3 — pepsine+ pancreatine

Comparing preparations "a" and "b" one can notice that in the case of digestion with lipase, without preliminary treatment with proteolytic enzymes, the preparation homogenised at 200 atm has its fat digestion degree by 12.8% higher compared with preparation "a" (70 atm.). On the other hand, when the preparation is preliminarily digested with proteolytic enzymes, the situation is reversed — the preparation homogenised at 70 atm. has a slightly higher digestibility than preparation "b" (200 atm.).

The data quoted above refer to the initial digestibility both of oil and of the preparations tested.

Analysing the changes in the digestibility of the soya bean oil and the fat in the protein-fat powders occuring during induction, one may observe that the highest digestibility when digesting with lipase (41%)

is displayed in the final stage by pure soya oil. Analogous changes for preparations "a" and "b" are 22.4% and 20.4%, respectively. This relatively high decrease of digestibility becomes smaller after the preliminary treatment with proteolytic enzymes, to be from 10.6% (pepsine) to 9.1% (pepsine+pancreatine) for preparation "a", and from 6.9% to 4.6%, respectively, for preparation "b".

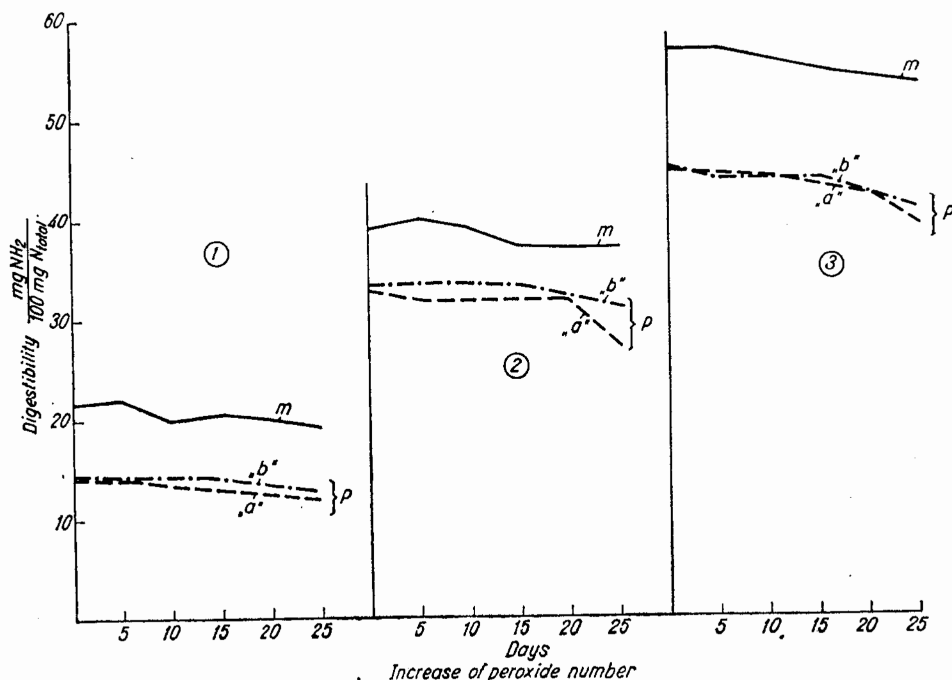


Fig. 3. Changes of protein digestibility; m — dry milk powder, p — preparations; 1 — pepsine, 2 — pepsine+trypsine, 3 — pepsine+pancreatine

Fig. 3. shows the data concerning changes in the susceptibility of proteins contained in the aforementioned preparations to the action of proteolytic enzymes. Analysing the results obtained, one can notice that in each of variants investigated, the digestibility of the protein component of the preparations was lower than the digestibility of initial milk dried by the atomisation method under identical conditions as the preparations were dried. Thus, the digestibility of the two preparations was lower than in the powder milk by:

35.6-40.1% when digested with pepsine,

13.9-16.2% when digested with pepsine and tripsine,

15.8-21.6% when digested with pepsine and pancreatine.

Comparing, in turn, the decrease in the digestibility of the milk powder and of the preparations, occuring under the effect of oxidised fat on protein in the induction period of the autooxidation process, one can state that in the beginning the decrease is rather small. More essential

changes can be observed only towards the end of this period, when the value of the peroxide number begins to grow rapidly. The decrease of digestibility is much more pronounced in the fatty-protein preparations (12.1%-15.9%) than in the milk powder (4.8%-10.8%) containing only some 1% of fat. The decrease of digestibility towards the end of the induction process is noticeable, however, mainly when the preparations

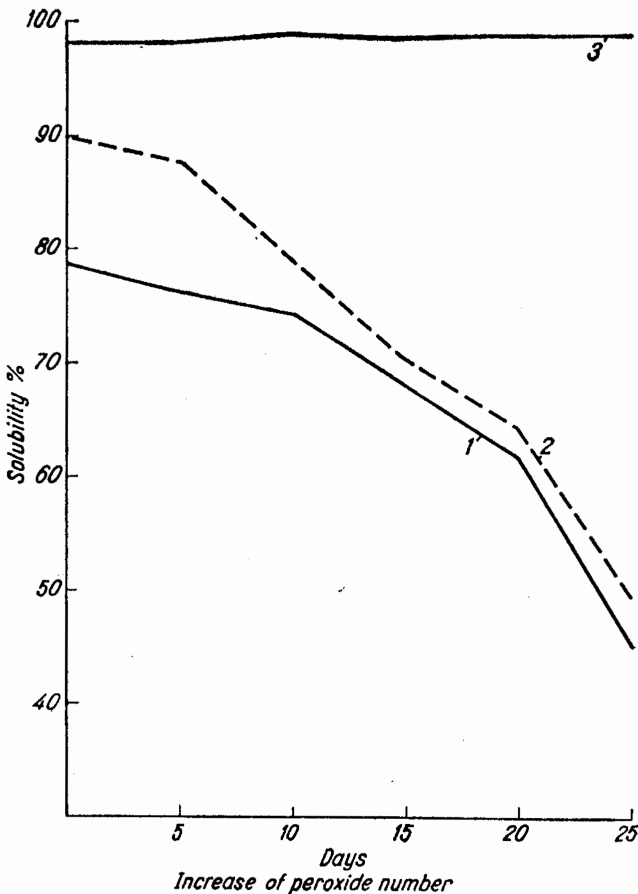


Fig. 4. Changes of preparations solubility; 1 — preparation before free fat extraction, 2 — preparation after free fat extraction, 3 — skim-milk powder

are digested with pancreatic enzymes. The dependencies observed are almost analogous to the skimmed and non-skimmed preparations, i.e. deprived of the so-called free fat but containing bound fat. In the light of these data it can be supposed that the latter form of fat decides about the decrease of digestibility of protein in preparations tested, compared to powdered milk.

To determine the eventual changes of the physical properties of the fat-protein preparations, their solubility degree was investigated as well and compared with the solubility of powdered milk stored under identical conditions (Fig. 5). The results obtained permit one to state that changes in the degree of solubility of the preparation are closely connected with the growth of the peroxide number of the free fat. In the initial phase of the induction period of the autooxidation process, the solubility of preparations changes only insignificantly. With the accumulation of peroxides, the decrease of solubility becomes more and more noticeable, its rapid drop occurring towards the end of the induction period; in the final stage of testing solubility decreases by some 57% of its initial value.

## CONCLUSIONS

1. The fat in the fresh preparations under test, compared to soya bean oil (intial raw material) features better susceptibility to the action of lipase. Initial treatment with proteolytic ensymes still increases this effect.

2. In the induction period the fat contained in the preparations displays reduced susceptibility to the action of lipase compared with the control soya oil sample. After preliminary treatment with proteolytic enzymes the "in vitro" decrease in the digestibility of fat in the preparations is low and does not exceed 10%.

3. The protein in the preparations tested features poorer susceptibility to the action of proteolytic enzymes compared with fresh powdered milk.

4. The decrease of the "in vitro" digestibility of the protein in milk powder and in the preparations being tested in the beginning of the induction period is low and it becomes more pronounced only towards the end of this period, accompanied by a rapid growth of the peroxide number.

5. The decrease of the degree of solubility of preparations tested is essential and is closely related to the growth of the free fat peroxide number, whereas the solubility of the control skimmed milk powder sample does not undergo practically any changes.

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## WPŁYW PROCESU AUTOOKSYDACJI PREPARATÓW BIAŁKOWO-TŁUSZCZOWYCH NA PODATNOŚĆ ICH NA TRAWIENIE ENZYMATYCZNE I ROZPUSZCZALNOŚĆ

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### Streszczenie

Określano zmiany strawności „in vitro" tłuszczu i białka oraz rozpuszczalności wysokoenergetycznych preparatów białkowo-tłuszczowych w czasie ich przechowywania. Materiał do badań stanowiły modelowe preparaty otrzymane z odtłuszczonego mleka (1% tłuszczu w s.m.) i rafinowanego oleju sojowego, które w postaci suchych proszków poddane były przyspieszonej autooksydacji (60°C i wymuszony przepływ powietrza). Preparaty różniły się tylko wartością ciśnienia stosowanego przy homogenizacji emulsji (preparat „a" — 70 atm., preparat „b" — 200 atm.). Celem pracy było uchwycenie zmian zachodzących przy stosunkowo słabym zaawansowaniu reakcji wywołanych działaniem tlenu, jak to ma miejsce w praktycznym przechowywaniu produktów zachowujących pełną przydatność konsumpcyjną. W pobieranych okresowo próbach preparatów oznaczano stopień strawności „in vitro" tłuszczu i białka poddając je działaniu lipazy, pepsyny, trypsyny i pakreatyny. Równolegle określano poziom tłuszczu wolnego i związanego oraz wartość liczb charakteryzujących stopień utlenienia frakcji lipidowej. Stwierdzono, że świeże, badane preparaty charakteryzowały się znacznie lepszą w stosunku do oleju sojowego (próba kontrolna) podatnością na działanie lipazy (rys. 2). Strawność oleju sojowego w warunkach doświadczenia wyrażała się liczbą 6,60, a dla preparatów „a" i „b" odpowiednio liczbami 15,6 i 17,6. Po wstępnym traktowaniu enzymami proteolitycznymi wartości wzrastały i wynosiły dla pepsyny 21,8 i 18,8 oraz 21,9 i 19,9 dla pankreatyny. W okresie indukcyjnym procesu autooksydacji podatność na działanie lipazy obniżyła się o 40% w przypadku oleju sojowego, w przypadku preparatów „a" i „b" o 22,4% i 20,4%. Po wstępnym trawieniu enzymami proteolitycznymi wartości te były niższe i kształtowały się dla preparatu „a" na poziomie od 10,6% (pepsyna) do 9,1% (pepsyna+pankreatyna), a dla preparatu „b" odpowiednio od 6,9% do 4,6%. Podatność białka na działanie enzymów proteolitycznych (rys. 3) była w przypadku obu preparatów mniejsza w stosunku do odtłuszczonego, wysuszonego rozpyłowo mleka o 35,6%-40,1% przy trawieniu pepsyną, 13,9%-16,2% przy działaniu pepsyną i trypsyną oraz 15,8%-21,6% przy trawieniu pepsyną i pankreatyną. Spadek

strawności białka zachodzący pod wpływem działania utlenionego tłuszczu na białko, zarówno w sproszkowanym mleku, jak i w preparatach był początkowo nieznaczny. Wyraźniejsze zmiany wystąpiły dopiero pod koniec okresu indukcyjnego. Podatność na trawienie proteolityczne obniżyła się (w stosunku do wartości początkowej) w preparatach o 12,1%-15,9%, w proszku mlecznym o 4,8%-10,8%. Zmiany stopnia rozpuszczalności preparatu (rys. 4) były ściśle związane ze wzrostem liczby nadtlenkowej tłuszczu wolnego, przy czym gwałtowne obniżenie wartości tego wyróżnika wystąpiło pod koniec okresu indukcyjnego (rys. 1). W końcowym etapie badań stopień rozpuszczalności preparatu był o ok. 57% niższy w stosunku do wartości początkowej.