ZESZYTY PROBLEMOWE POSTĘPÓW NAUK ROLNICZYCH 1995 z. 427, 115-120

CHARACTERISTICS OF LIPOLYTIC ENZYMES OF RAPESEEDS*

J. Grabska, L. Jędrychowski, D. Mierzejewska

Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, 10-718 Olsztyn, Poland

A b s t r a c t. Some properties of lipolytic enzymes of a microsomal fraction of two Polish varieties of rapeseed were qualified. Enzymes extracts were prepared from germinated rapeseeds of two varieties: double improved - Bolko and high erucic - Skrzeszowicki. The lipase was purified by ultracentifuge. The obtained supernatant was used to chracterise the lipase and further to purify by a column chromatography method, using the following carriers: Sephadex G-100, Sephacryl S-300, DEAE sephadex A-50 and Q Sepharose.

Examined enzymatic extracts showed two optima of lipolytic activity: pH 7.5 and pH 9.0. The highest degree of hydrolysis of tributyrylglicerol (TC4:0) revealed lipases of the Bolko variety seeds at 30 °C, and lipases of the Skrzeszowicki seeds at 40 °C. At the temperature 30-40 °C lipases maintained approx. 89 % to 93 % of their maximum activity. However, at 25 °C and 50 °C their activity was reduced to approx 20 %. When the temperature was above 40 °C, thermal inactivation of lipolytic enzymes from two varieties progressed in two stages. Examined lipases maintained 20 % to 30 % of their initial activity, after storing them for 1 h at 60 °C. During the purification of lipases, carried out by a column chromatography method, the most favourable results were obtained using molecular filtration on Sephadex G-100 gel.

K e y w o r d s: rapeseed lipase, purification, characterisation, rapeseed

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, E.C.3.1.1.3) are the first activating cell enzymes during the germination of seeds. Because of their activity, released fats are transformed into sugars, essential energetistic components during plant growth. Examinations of lipase properties and the location of lipases in cell structures of oilseed plants have been conducted for some years. They showed that native lipases are located in a microsomal fraction in glyoxysomes (peanuts, castor bean - alkaline lipase, soya) [1,3,5,7]. Examinations of rapeseeds carried out so far indicated that from 75 % to 80 % of lipolytic activity is located in a microsomal fraction. Whereas, 20-25 % of this activity has a relation with a membrane of lipid bodies.

The aim of the research was to obtain lipolytic enzymes from the microsomal fraction of two Polish varieties of rapeseed, their purification and determination of the selected biochemical properties.

MATERIAL AND METHODS

Enzymatic extracts were prepared from seed leaves of rapeseeds (germinated for 5 days) of two varieties: double improved Bolko and high erucic - Skrzeszowicki according to Lin's and Huang's method [7]. Isolation of lipases was conducted according to the Hills's and Murphy's [4,8] method and Hassanien's and Mukherjee's method [3,9] using ultracentrifugation: at acceleration 100 000 g for 90 min and at 23 000 g for 30 min. A supernatant

*This work was supported by the State Committee for Scientific Research, Poland, under grant No. 5 S306 010 06

obtained after ultracentrifugation at acceleration 23 000 g for 30 min was used for the initial characterisation of lipases.

Lipases purification was carried out by a column chromatography method using the following carriers: Sephadex G-100, Sephacryl S-300, DEAE Sephadex A-50 and Q Sepharose.

Molecular filtration on Sephadex G-100 and DEAE Sephadex A-50 gel was carried out by a traditional column chromatography method, while on Sephacryl S-300 and Q Sepharose gels the FPLC (PHARMACIA) technique was used. The separation conditions were established based on our own initial researches and other authors' examinations [1,8,10].

The protein content was measured by a spectrophotometric method. The measurements of absorbance were conducted at the wavelength 280 nm and estimated in mg/ml. The standard solution of rapeseed albumin protein was a standard.

Lipolytic activity was measured by the diffusion method according to Lawrence *et al.* [6] and during lipase characterisation, additionally by a titration method at constant pH, using the automatic titration set of RADIOMETER firm (Denmark).

Lipolytic activity changes of examined enzymes, after different time of thermostating at temperatures from 20-22 °C (room temperature) to 60 °C, were measured by a diffusion and titration method at constant pH.

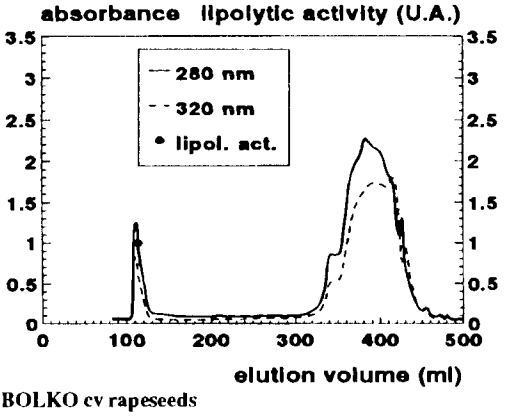
Emulsion of tributyrylglycerol (TC4:0) was used as a substrate. Results were expressed in conventional activity units (J.A.) or in J.A./mg of protein (the specific activity) and equivalents FFA/min/ml of enzymatic extract.

RESULTS

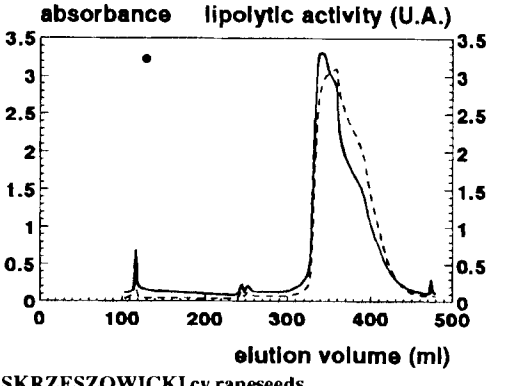
The ultracentrifugation (100 000 g x 90 min) of the enzymatic extract, for the first isolation and rapeseed lipases purification produced three fractions: lipid bodies, supernatant and pellet. The lipolytic activity of supernatants containing mainly microsomal cell enzymes, was approx. 92 % to 96 % of the total lipolitic activity. The rest of fractions revealed only approx. 2 % to about 5 % of the total activity of lipolytic enzymes. The ultracentrifugation process produced approx. 3 to 4 times greater increase of specific activity of the lipases examined. All the obtained fractions were subjected to further purification by molecular filtration on Sephadex G-100 gel using for 50 mM elution the TRIS-HCl buffer of pH 7.5.

Following separation of the supernatant of both rapeseed varieties, two main fractions were obtained. Lipolytic activity was found for fractions with the highest molecular weight (Fig. 1).

As a result of the separation of the lipid bodies fraction on the Sephadex G-100 gel two different chromatograms for both rapeseed varieties



BOLKO cv rapeseeds



SKRZESZOWICKI cv rapeseeds
Degree of purification as compared to specific lipolytic activity before: separation - 14; ultracentrifugation - 56

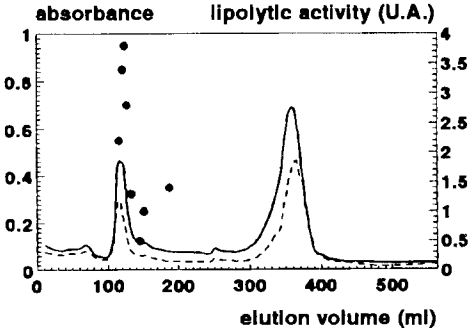
Fig. 1. Separation of supernatant fraction (100 000 g) of enzyme extracts. G-100 Sephadex.

were obtained. However, no hydrolysis activity was found in any fraction. Because of the molecular filtration on the gel Sephadex G-100 of the pellet from Bolko and Skrzeszowicki enzymatic extracts, a similar profile of protein fractions was obtained. Hydrolysis TC4:0 was revealed only in fractions with the highest molecular weight, and their specific lipolytic activity was approx. 10-17 times greater than the lipolytic activity of the specific sample before chromatographic separation.

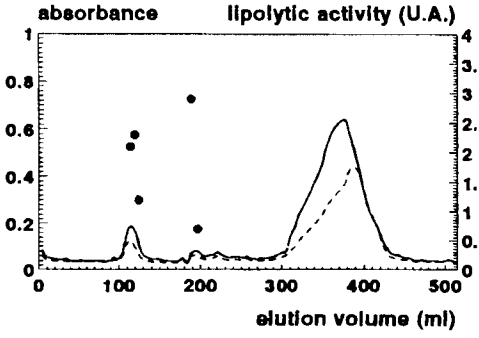
During the second way of isolation and purification of lipolytic enzymes from rapeseeds, ultracentrifugation of enzymatic extracts at acceleration 23 000 g for 30 min was used. The obtained supernatants contained microsomal enzymes of specific activity 3-4 times higher than the lipolytic activity of the extracts before ultracentrifugation.

Further purification of these enzymes by molecular filtration on the Sephadex G-100 gel, produced two peaks, like those produced during supernatants separation, after ultracentrifugation at acceleration 100 000 g. The second peak was a mixture of inseparable protein fractions. Fractions with a very high molecular weight (with elution about V_o of deposit) presented the ability of tributyrylglycerol hydrolysis, and their specific lipolytic activity was some or anywhere from ten to twenty times higher than lipases specific activity prior to chromatographic separation (Fig. 2). Similar chromatographic pictures were obtained during separation of the same supernatants on Sephacryl gel S-300. Only protein fractions with a high molecular weight revealed lipolytic activity. The degree of lipases purification indicated by the ratio of the lipolytic activity of each fraction after separation to the same fraction but prior to separation, was from about 3 to about 20 (Fig. 3).

Applying the ion-exchange chromatography on gel DEAE - Sephadex A-50 for purifying the examined lipases allowed the more favourable separation of protein compounds from phenolic ones, than by molecular filtration method on Sephadex G-100 gel. Lipolytic activity was revealed in fractions within the first peak, eluting at



BOLKO cv rapeseeds
Degree of purification as compared to specific lipolytic activity before Sephadex separation: from 11 to 24



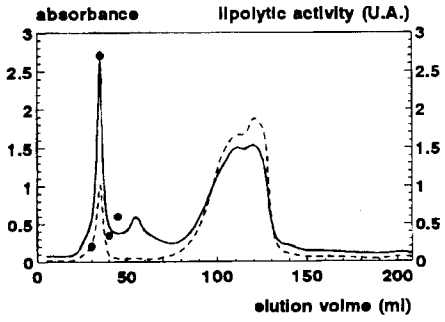
SKRZESZOWICKI cv rapeseeds
Degree of purification as compared to specific lipolytic activity before Sephadex separation: from 31 to 38

Fig. 2. Separation of supernatant fraction (23 000 g) of enzyme extracts. G-100 Sephadex. Explanations as in Fig. 1.

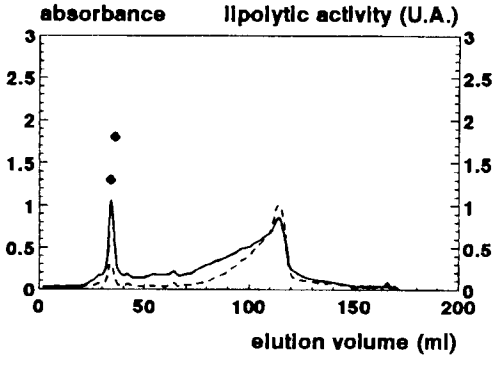
0.2 M concentration of NaCl (Fig. 4).

Chromatogram of microsomal lipases separation while using Q Sepharose gel indicated the presence of many protein fractions. Activity of lipolytic enzymes was located in fractions of high protein content, eluting at a concentration of NaCl from 0.3 M to 1 M.

Fractions, after microsomal separation of lipases on Sephadex G-100 gel indicating the ability of tributyrylglycerol hydrolysis, were lyophilized and applied as an antigen for obtaining the polyclonal antibodies of native rapeseed lipase. The obtained antibodies will be used to examine of native lipase of rapeseeds using immunological methods (ELISA, immunoblotting).

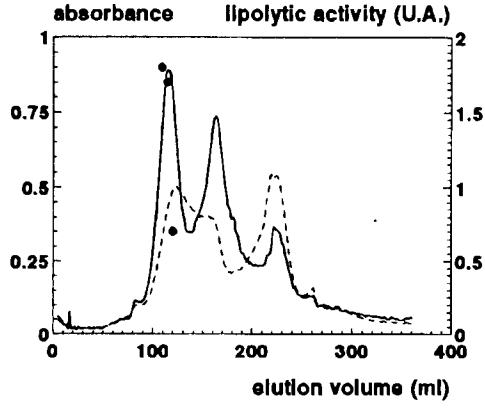


BOLKO cv rapeseeds
Degree of purification as compared to specific lipolytic activity before Sephacryl separation: from 3 to 4



SKRZESZOWICKI cv rapeseeds
Degree of purification as compared to specific lipolytic activity before Sephacryl separation: from 6 to 20

Fig. 3. Separation of supernatant fraction (23 000 g) of enzyme extracts Sephacryl S - 300. Explanations as in Fig. 1.



DEAE Sephadex A-50

Fig. 4. Separation of supernatant fraction (23 000 g) of enzyme extracts of Bolko cv rapeseeds. Explanations as in Fig. 1.

Lipases characteristics

Examined fraction of rapeseeds microsomal lipase revealed two optima of lipolytic activity in pH 7.5 and 9.0. It can indicate non-homogeneity of an enzyme in microsomal fraction or lipases remainder of different properties from other cell organelles (for example lipid bodies) [3,4,7]. Obtained results within this range are in accordance with the results of Hills and Murpy [4], which indicated presence of two lipases: microsomal fraction and lipid bodies membrane of following pH activity optimums: 7.5 and 9.0. Before, Lin and Huang [7] suggested the presence of one lipase in rapeseeds. Moreover, in mentioned researches' opinions, microsomal membranes contained lipase, present in supernatant fraction after enzymatic extracts ultracentrifugation, are simply remainders of lipid bodies membranes after hydrolysis of reserve tricacyloglyceroles. The complexity of enzymatic proteins observed during isolation and purification of enzymatic extracts (very high molecular weight of proteins) may either indicate the non-homogeneity character of these enzymes.

These results differ from our earlier examinations [2], in which the optimum characteristic for lipolytic activity of enzymatic extracts of both varieties of rapeseeds, measured by diffusion method in comparison with TC4:0, was 8.5 pH. Whereas, during the measurement of lipolytic activity by extractive-titrative method using rapeseed oil, the optimum lipases activity of the Skrzeszowicki variety seeds was pH 7.5 and of Bolko variety - pH 8.5. Observed differences could be due to the different degree of lipases purification.

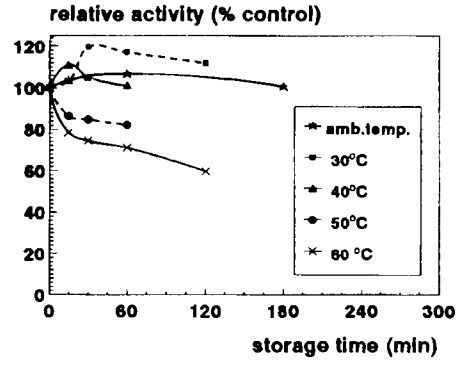
The highest hydrolysis degree of tributyrylglycerol (TC4:0) was observed at the temperature of 30 °C for lipases of the Bolko variety and 40 °C for lipases of Skrzeszowicki variety.

At 30-45 °C temperature examined lipases maintained from about 89 % to about 93 % of their maximum activity, and at the temperatures of 25 °C and 50 °C their activity was reduced about 20 %.

Examining the changes of lipolytic activity within the temperature interval from 20-22 °C (room temperature) to 60 °C, it was indicated that inactivation of microsomal fractions measured by diffusion method at temperatures above 40 °C is conducted in two stages: during the first 30 min of storing at these temperatures there was a rapid decrease of enzymatic activity, and after this time the speed of enzyme inactivation was reduced, while maintaining from about 40 % to about 60 % of activity after 2 and 3 h activity of temperature 60 °C (Fig. 5).

Changes of lipolytic activity of the same enzymes subjected to the same temperature-time conditions, measured by titration method at the same pH, show greater enzymes thermolability from the Bolko seed variety at temperatures from 30 to 50 °C.

After 1 h of storing at temperatures 30, 40 and 50 °C, lipases from the Bolko rapeseeds variety maintained about: 60, 55 and 40 % of their initial activity. Whereas lipases of the Skrzeszowicki seed variety revealed about 90, 80 and 70 % of their initial lipolytic activity. At 60 °C lipases of both rapeseed varieties had similar thermolability. After 1 h of storing at this temperature they were losing from about 70 % to about 80 % of their initial activity (Fig. 6).



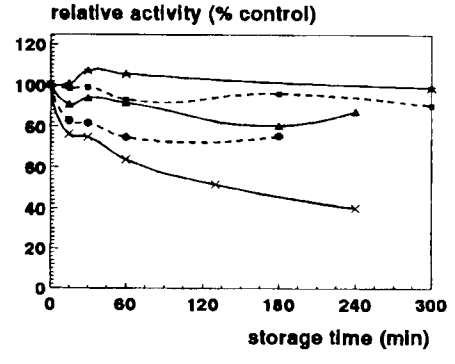
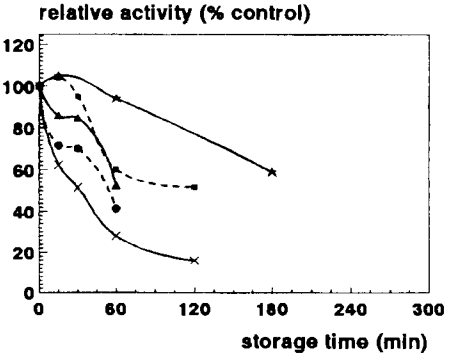


Fig. 5. Thermostability of lipolytic enzymes of rapeseeds supernatant 23 000 g examined by diffusion method.



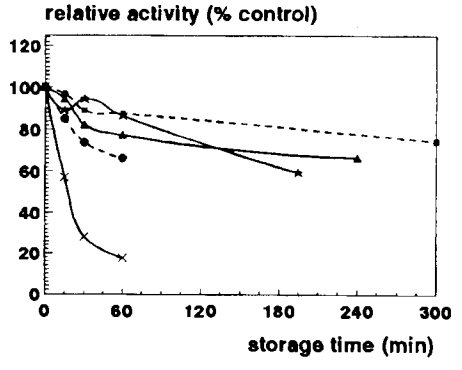


Fig. 6. Thermostability of lipolytic enzymes of rapeseeds supernatant 23 000 g examined by titrimetric assy method. Explanations as in Fig. 5.

CONCLUSIONS

Among applied methods of isolation and lipases purification the most favourable results were obtained using ultracentrifugation of enzymatic

extracts, then molecular filtration of obtained supernatants on Sephadex G-100 gel.

Lipolytic activity presence in fractions of high molecular weight (about 2 000 kD) indicates the complex character of examined lipases protein.

Conducted characterisation of rapeseeds lipases reveals their non-homogeneity character.

REFERENCES

1. **Fuchs C., Hansen G.:** Partial purification and some properties of *Brassica napus* lipase. Z. Naturforsch, 49, 293-301, 1994.
2. **Grabska J., Jędrychowski L.:** Methods of lipolytic activity estimation in the seed of oilseed (in Polish). Post. Nauk Roln., 6, 143-150, 1993.
3. **Hassanien F.R., Mukherjee K.D.:** Isolation of lipase from germinating oilseeds for biotechnological processes. JAOCS, 63(7), 893-897, 1986.
4. **Hills M.J., Murphy D.J.:** Characterization of lipases from the lipid bodies and microsomal membranes of erucic acid-free oilseed rape (*Brassica napus*) cotyledons. Biochem. J., 249, 687-693, 1988.
5. **Huang A.H., Lin Y., Wang S.:** Characteristics and biosynthesis of seed lipases in maize and other plant species. JAOCS, 6, 897-899, 1988.
6. **Lawrence R.C., Fryer T.F., Reiter B.:** Raid method for the quantitative estimation of microbial lipases. Nature, 1264-1265, 1967.
7. **Lin Y., Huang A.H.:** Lipase in lipid bodies of cotyledons of rape and mustard seedlings. Archiv. Biochem. Biophys., 225(1), 360-369, 1983.
8. **O'Sullivan J.N., Hills M.J., Murphy D.J.:** Purification and properties of lipase from oilseed rape (*Brassica napus* L.). Catabolism of lipids. Biochem. J., 313-315, 1990.
9. **Price N.C.:** Enzyme assays. In: A Practical Approch (Eds R. Eisenthal, M.J. Danson). Oxford University Press, Oxford-New York-Tokyo, 255-273, 1993.
10. **Weselake R.J., Thomson L.W., Tenaschuk D., MacKenzie S.L.:** Properties of solubized microsomal lipases from germinating *Brassica napus*. Plant Physiol., 91, 1303-1307, 1989.

CHARAKTERYSTYKA ENZYMÓW LIPOLITYCZNYCH NASION RZEPAKU

Określono właściwości enzymów lipolitycznych frakcji mikrosomalnej nasion dwóch polskich odmian rzepaku. Ekstrakty enzymatyczne przygotowywano ze skiełkowanych nasion rzepaku dwóch odmian: podwójnie ulepszonej - Bolko i wysokoerukowej - Skrzeszowicki. Lipazę oczyszczano przez ultrawirowanie, a uzyskany supernatant zastosowano do scharakteryzowania lipazy i dalszego oczyszczania metodą chromatografii kolumnowej z wykorzystaniem następujących nośników: Sephadex G-100, Sephacryl S-300, DEAE Sephadex A-50 i Q Sepharose.

Badane ekstrakty enzymatyczne wykazywały dwa optima aktywności lipolitycznej: pH 7.5 i pH 9.0. Najwyższym stopniem hydrolizy tributyryloglicerolu (TC4:0) charakteryzowały się lipazy z nasion odmiany Bolko w temperaturze 30 °C, zaś lipazy z nasion odmiany Skrzeszowicki w temperaturze 40 °C. W przedziale temperatur 30-45 °C lipazy zachowywały od ok. 89 do 93 % swojej aktywności maksymalnej, a w temperaturach 25 i 50 °C ich aktywność zmniejszała się o ok. 20 %. W temperaturach powyżej 40 °C inaktywacja termiczna enzymów lipolitycznych obu odmian przebiegała dwufazowo. Badane lipazy zachowywały 20 % do 30 % aktywności początkowej po 1 godzinie przetrzymywania w temperaturze 60 °C. Podczas oczyszczania lipaz metodą chromatografii kolumnowej, najkorzystniejsze wyniki uzyskano stosując sączenie molekularne na żelu Sephadex G-100.

S l o w a k l u c z o w e: lipaza rzepaku, oczyszczanie, charakterystyka, rzepak.