Biolog. Journal of Armenia, 1 (67), 2015

STUDY OF SOME PROPERTIES OF ENDOINULASE ENZYME OF DAPPLE

G.Y. BAGHDASARYAN

Goris State University, Faculty of Physical-Pedagogical Specialties,
Department of Biology and Chemistry
baghdasaryan.grigor.1959@mail.ru

The extracellular endoinulase synthetized in *Taraxacum officinale* root cells has been released, solved in water and settled at pH=6.2 then treated by butanol. The fractionation was obtain-ned by butanol and acetone gel-filtration as well as a column G-150 sefadex (with 2.0×160 cm sizes). The thermal threshold of activity preservation was 45^{0}C-60^{0}C. Under these thermal condi-tions the enzyme may preserve its activity during 5-7 days. The values of K_m and v_{max} were deter-mined.

Dapple – inulin – endoinulase – fructose – glucose

Taraxacum officinale-ի արմատային բջիջներում սինթեզված արտաբջջային էնդոինուլազը անջատվել, լուծվել է ջրում և նստեցվել pH=6.2-ում, ապա մշակվել բութանոլով: Կատարվել է ֆերմենտի ֆրակցիոնացում բութանոլ և ացետոն ժել-ֆիլտրացիայի մեթոդով, ինչպես նաև G-150 սեֆադեքսով աշտարակի միջոցով (2.0□160 սմ չափերով): Ակտիվության պահպանման ջերմային շեմը կազմել է 45^{0}C-60^{0}C: Այս ջերմային պայմաններում ֆերմենտը կարող է պահպանել իր ակտիվությունը 5-7 օրվա ընթացքում: Որոշվել են K_m-ի և v_{max} արժեքները:

Խատուտիկ – ինուլին – էնդոինուլազ – ֆրուկտոզ – գլյուկոզ

Внеклеточную эндоинулазу, синтезированную в корневых клетках *Taraxacum officinale,* выделяли, растворяли в воде и осаждали при pH=6.2, затем обрабатывали бутанолом. Проводили фракционирование методом гель-фильтрации, с применением бутанола и ацетона, а также колонки с G-150 сефадексом (с размерами 2.0□160 см). Термальный порог сохранения активности составлял 45^{0}C-60^{0}C. При таких условиях фермент может сохранить свою активность в течение 5-7 мин. Определяли значения K_m и v_{max}.

Одуванчик – инулин – эндоинулаза – фруктоза – глюкоза

The issue on biological condition improvment is the most important topic worrying the population. The ways should be found to facilitate, carry and provide qualitatively new degree of the value of food. From this point of view the best raw material among tubers are artichokes (*Helianthus tuberosus*). The tubers contain inulin polysaccharide which is mainly consisted of digestible mono-sugar fructose the amount of which according to obtained data is up to 90% [11-13].

The mentioned plant considered to Compositae family has a high harvest of tubers and green mass, is not demandable to external medium conditions. It may persist severe conditions and is not infectable, besides it is the best biological protector of surrounding – cleans the medium from dangerous gases, enriches by oxygen [1]. Many investigators [10-14] mention about the significance of inulin in the region of enhancing of food quality and value. The authors give data concerning to inulin effect in intestines, particularly in colon where it effects on digestion process, as well as exchange processes of proteins, carbohydrates and fat. Besides it is shown that inulin supports the growth of microorganisms in intestines, decreases the possibility of intestine polyp formation and cancer. Inulin decreases the amount of sugar and cholesterol in blood as well [5].

In large amounts inulin is used in different regions of population economy, food industry, confectionery, medicine, pharmacology, agriculture and etc. That is why the obtaining of inulin in industrial scales is an important topic in big countries. Taking it into account the demand of pure inulin is increased.

The aim of present paper is to obtain the inulin from dapple (*Taraxacum offcinale*) and to characterize biological, biochemical and physical-chemical properties of pure enzyme – endoinulase.

Materials and methods. As an investigation object dapple was used (*Taraxacum office-nale*) which is gathered from meadows. This plant grows in the territory of Armenia everywhere especially in mount zones. According to our investigations the dapple growing in meadows possesses higher enzymatic (inulase) activity [3]. Dapple may be grown in laboratory conditions during the whole year and is not demandable to medium conditions. It is also indifferent to azotic fertilizer. According to our data the addition of ammonia with amount of 1/10 norm of azotic fertilizers given to herbs in ground, the activity of inulase enzyme increases several times, but growth and development of the herb decrease by 15-20 days. In wild (gardens, meadows, edges of highways) inulase gets to high activity during herb bud formation and flowering [2,3].

To determine inulase activity the roots of dapple gathered from meadow were washed by faucet water, the core of root was removed and dried in room conditions, crushed by knife, mashed in mortar until obtaining of homogeneous puree and weighed by analytic weight, and then protein amount was measured in basic extract by Lowry method [6]. The enzymatic preparation was 15g, from which 5g basic material was separated and dissolved in 0.1M sodium acetate buffer (pH 5.5). The dialysis was made by semi-transparent membrane. Proteins are not diffused via the membrane. In this time low molecular compounds enter into external medium.

Inulase activity in basic extract was determined as reported [7]. The basic extract was treated by butanol then centrifuged by 10000g/20min. Supernatant was used as basic enzyme and the whole process of purification was released with supernatant.

Enzyme obtaining: Intracellular inulase enzyme obtained from dapple was fractionated by gel-filtration method using sefadex G-150 (2.0□120cm). Fractions with 2ml volume were gathered and stream rate was 50ml/h. The gathered mass was centrifuged, and the sediment was purified by 80% butanol. This is the treatment by butanol. For enzyme sedimentation butanol and acetone solvents with different densities were used.

The obtained data show that at gel-filtration by G-150 sefadex the optimum of protein amount is in interval of the 2nd and the 3rd fractions and its amount decreases, but the little protein amount is revealed until the 23rd fraction. The peak of inulase enzyme is revealed in the 12th and the 13th fractions then sharply decreases in the 16th fraction, after 17th and 18th fractions inulase activity is not revealed. The whole quenching process was carried out with enzyme active fractions.

On the next step enzyme purification was carried out with cold acetone (-10^{0}C, 60% acetone), since in acetone stored in room conditions the specific activity of inulase enzyme decreases lightly. After centrigugation supernatant was dissolved in 45% acetone (-10^{0}C). Settling by $(NH_4)_2SO_4$ was not carried out taking into account the fact that inulase or □-D-fructo-furanozidase [EC 3,2.1.26] released from microorganisms are well purified by organic

solvents [15,16]. Continuing inulase purification process by cold acetone on the next steps 71.3 times purified inulase with 15% outcome was obtained. The studies of enzyme biochemical and physicochemical properties were carried out on pure preparations.

Determination of molecular mass: To determine purified enzyme mass (molecular mass) marker proteins were used (Sigma). It was determined via relative mobility [9].

Results and Discussion. pH optimum of inulase enzyme of dapple: The aim of the next group of experiments was to reveal pH optimum of inulase enzyme functioning. pH optimization was done by sodium phosphate buffer (6.2-8.0). The results of these studies are presented in tab. 3. It is seen from tab. 3 that inulase enzyme pH optimum is 6.2, higher of it as well as less of it the functioning criteria are low. Thus, if at pH 6.2 there are 362.0 mmol/l and 1.3 mmol/l criteria for fructose and glucose respectively, at pH 5.4 and 7.4 there are 300 mmol/l and 233 mmol/l fructose. Besides settling by basic extract (precipitation) the same was done by cold acetone solutions presented in tab. 3.

Intracellular inulase enzyme from dapple was purified by gel-filtration method with some modifications [4]. The results are presented in tab. 1. Centrifugation was carried out during 15 min by 10000g, fractions were 2 ml (50 ml/h rate) and the purification was continued by the same way.

Table 1. Inulase enzyme purification process of dapple (*Taraxacum officinale* Wigg) n=4

Fractions	Volume ml	Enzyme concen-tration unit/ml	Whole amount of units ×103	Protein concent-ration mg/ml	Specific activity unit/mg	Out come %	Purifica-tion degree
Basic extract	12000	205	2460	13.5	17	100	1
Settling pH=6.2	5000	216	1080	4.97	42	44	2.47
Treatment by butanol	4450	166	739	1.18	141	30	8.3
Settling by 60% cold acetone	55	2623	680	12.42	211.2	28	12.42
Settling by 45% cold acetone	20	3324.41	525	6.88	483.2	21	28.4
Settling by 40% cold acetone	20	69120	378	5.7	1212	15	71.35

It is obvious from tab. 1, in pH=6.2 buffer and refrigerator after 1 day and centrifugation with 20 min duration the enzyme is purified about 2.5 times, and after butanol treatment 8.8 times purified preparation is obtained.

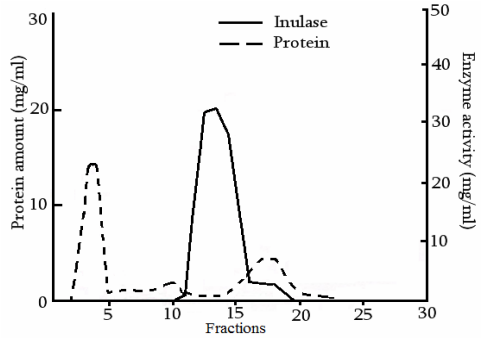

Fig. 1. The amounts of protein (mg/ml) and enzyme activity (mg/ml) in different fractions.

According to fig. 1, protein fractions were filtrated since the 2nd fraction and the peak is obtained in the 4th, 5th fractions and is continued until the 23rd fraction in that case, when for protein determination in each test tube 3-5ml filtrate was gathered, but enzyme activity is shown from 10th fraction and the peak is obtained in 13-15 fractions. In this case to determine inulase activity in each test tube 0.5ml eluent (filtrate) is gathered. Enzyme
purification was carried out in fractions containing enzyme activity. For inulase molecular mass determination the following proteins with known molecular masses were used: fibri-nogen – 330 kDa, catalase – 240 kDa, aldolase – 160 kDa, albumin – 45 kDa and cyto-chrome C – 12.3 kDa. Based on protein mobility inulase molecular mass was determined which is equal to 176.5 kDa.

The next group of experiments is devoted to temperature effect on inulase activity. The results are presented in tab. 2.

As it is obvious from tab. 2, inulase optimal activity was revealed and it was equal to 365.0 mmol/l fructose which is considered to be 100%. With increasing of temperature the activity decreases gradually and at 60^0C is equal to 5% of total activity.

Table 2. Temperature effect on enzyme (inulase) activity n=4

Temperature ^{0}C	Inulase activity %	Fructose amount mmol/l	Fructose amount %
15	20.0	235.0	64.4
20	23.5	257.2	70.5
25	31.2	285.0	78.0
30	40.0	300.0	82.2
35	52.3	307.7	84.3
40	65.0	325.6	89.2
45	100	365.0	100.0
50	89.0	267.0	72.7
55	16.0	280.5	76.8
60	5.0	233.0	63.9

Table 3. Inulase enzyme pH optimum of dapple n=4

pH value	Inulin %	Fructose mmol/l	Glucose mmol/l	Fructose % in extract	Glucose % in extract
5.4	10.7	300.0	2.2	99.3	0.7
5.8	-	307.7	1.1	99.6	0.4
6.2	-	362.0	1.3	99.6	0.4
6.6	-	263.0	1.6	96.4	0.6
7.0	-	286.0	3.3	98.8	1.2
7.4	-	233.0	2.4	99.0	1.0

It is interesting that at hydrolysis by dapple inulase the percentage of fructose to glucose remains higher from 99%, which is sufficiently high criterion and is useful at getting of syrup in industry since glucose amount in mixture, is always in 0.4-1.2% interval which is in permission frames, that is why this enzyme obtained from dapple gets a big advantage. In literature there are no an enzyme possessing such properties. The described enzymes were obtained from different microorganism groups [5, 8, 14].

Fructose accumulation dynamics (mmol/l) depending on enzyme concentration: The next group of experiments is devoted to enzyme concentration effect on fructose accumulation. This criterion is important for characterization of enzyme properties, since physiological conditions (pH) are prominent factors providing enzyme functioning rate in temperature and salt concentration medium.

The effect of these factors is presented on fig. 2. The presented curve obviously shows that with enzyme concentration increasing fructose accumulation in physiological medium (pH=6.2) and enzyme functioning in optimal temperature until enzymatic preparation addition obtained from dapple (20 ml) increase. Thus, fructose accumulation at preservation of physiological medium (pH=6.2) and other factors entirely depends on inulin concentration (up

20ml). 20ml concentration probably inhibits inulin functioning consequently fructose accumulation in medium.

Purified inulase substrate specificity to inulin was investigated and it was revealed that the value of K_m is low and equal to 0.22 mM, but V_{max} – relatively high – 293 mM/mg.

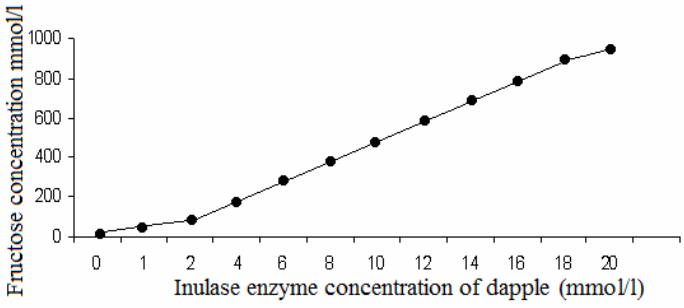

Fig. 2. Fructose accumulation dynamics (mmol/l) depending on enzyme concentration.

The partial purification of inulase enzyme (endoinulase) in natural conditions (from dandelion growing in meadows) by sefadex G-150, applying gel-filtration was presented. Enzyme settlement by buthanol and cold acetone was carried out which makes possible to obtain 71.3 times purified inulase with 15% outcome. The enzymatic preparation has also other advantages that are presented in this work. Dandelion may be grown in laboratory conditions during all seasons of year.

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Received on 06.10.2014