ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Biocatalytic Properties and Substrate Specificity of Proteinase Preparations From Different Sources.

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ABSTRACT

The comparative evaluation of biochemical and physical- and chemical properties of enzymatic complexes of proteolytic preparations from different sources was carried out. Kinetic constants of the enzymatic catalysis fractions of globular and fibrous proteins of meat raw materials were calculated. The results are of primary importance for the use of biochemical and technological effects of exogenous biocatalysis under the influence of proteolytic enzyme complexes action.

Keywords: proteolytic agents, enzyme complexes, collagen, kinetic constants, enzyme catalysis, proteolytic activity, collagenolytic activity.

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ISSN: 0975-8585

INTRODUCTION

In the field of industrial application of enzymatic preparations the meat industry concedes significantly to the industries of agrarian- and industrial complex processing raw materials of vegetable origin: alcohol manufacture, brewing industry, production of juice and fruit beverages, etc. Thus, unlike predominantly carbohydrate hydrolysis problem, to a lesser extent - carbohydrate and protein substrates, the problem of proteins and strengthened structure protein systems modification is of great importance for the meat industry [1,2].

Its decision is interrelated with the increase of biological and technological functionality of collagen-containing raw materials, partial or full replacement of traditional raw materials, improvement of the resulting products properties due to molecular conversion of proteins and complex biological systems transformation. At the same time, practical realization of enzyme engineering methods in the solution of separate problems of meat products technology is limited by two factors: on the one hand, it is the absence or limited industrial output of required specificity enzyme preparations, and on the other hand , it is a lack of scientific and reasonable recommendations about their application.

For the effective use of a hydrolytic enzyme preparation in meat products technology its specificity to hydrolysis of protein fractions of meat raw materials is greatly important. They differ in multicomponent structure and a variety of biological functions. So, when receiving protein hydrolyzers where an ultimate goal is the maximum destruction of proteins primary structure irrespective of a type, the most effective is the application of enzymes of a wide action range. This will be the main criterion of a choice along with the high level of activity [3,4].

In the solution of a problem of any protein fraction selective hydrolysis the degree of substrate specificity prevails over all the other indicators [5].

The purpose of the work is to give a comparative assessment of kinetic constants of enzyme hydrolysis of fractions of the globular and febrile proteins of meat raw materials, for practical use of biochemical and technological effects of an exogenous biocatalysis.

The objects of study were enzyme preparations of a microbic origin (Protosubtilin G10x, Megaterin G10x), and hydrobionts (the Kamchatka crab hepatopancreas) which choice is based on a comparative assessment of the biochemical properties of the enzyme complexes of the known producers in relation to processing of collagen-containing raw materials of the meat industry (tab. 1). The choice of enzyme preparations for comparison (papainase, trypsin) is caused by their known properties, and therefore, by the possibility of their use as standards [6-8].

Table 1: Biochemical characteristics of preparations enzyme complexes.

Enzyme preparation	Source, producer	Activity		Action optimum	
		PA, unit/g	KA, unit of optical density/mg	acid, units pH	thermal, ^{0}C
Protosubtilin G10x	*Bacillus subtilis,* JSC "Sibbiofarm"	100	0,17	7,0-7,2	40
Papain	*Papaya tree latex,* Biozym gezellschaft fur Enzymtechnologie, Germany	150	0,12	5,0 – 7,2	37 - 40
Trypsin	Pancreas, CJSC "Plant of endocrine enzymes"	240	0,01	7,0 - 9,0	50
Megaterin G20x	Basillus Megaterium, Laboratory sample	700	0,07	7,0-7,2	37-40
Food collagenase	the Kamchatka crab hepatopancreas,CJSC “Bioprogress”	125	0,30	7,0	38-40

Experiment conditions

The general proteolytic activity (PA) of enzyme preparations was determined by Anson's modified method [9] with the use of casein as a substratum on Gammersten at pH 7,2. For the PA unit we took the

amount of enzyme which in 1 minute at 30^0 C catalyzed transition in nonprecipitating by trichloroacetic acid condition quantity of casein by Gammersten containing 1 micromol of tyrosine.

The collagenolytic activity (CA) - according to the contents of oxyproline in the mixture, having formed as a result of enzyme action on native collagen in phosphatic buffer solution at 37 ^oC, pH 7.2, an enzyme to a substratum ratio - 1:1000, the durations of mixture incubation is 18 h [13].

The substratum for determination of collagenolytic activity was the purified collagen from cattle Achilles tendon. For its obtaining the tendon was purified of muscular fabric, fat and covers by a knife then cooled and minced on a gyroscope. The resulting mass was frozen, freeze-dried, ground in a colloid mill and suspended with the with NaOH solution of 0.1 mol/dm3 molar concentration (65 cm3 per 1 g of the dry mass) to dissolve the foreign proteins. Suspension was divided in the centrifuge, the sediment was washed out with NaOH solution with the molar concentration of 0,1 mol/dm3 and then it was centrifuged. The sediment was suspended in the distilled water and neutralized by 0,1 mol/dm^3 HCl solution, centrifuged and the collagen was washed out with water. Then the collagen was dissolved in 0,01 mol/dm^3 of hydrochloric acid solution. Viscous solution was separated with centrifugation from the undissolved particles and neutralized with NaOH solution with molar concentration of 0,1 mol/dm^3. The sediment collagen flakes were separated by centrifugation and washed with distilled water. Collagen sediment was washed out with ethanol and ether and then air dried.
The kinetics of enzymatic catalysis and substrate specificity of enzymes assessment were conducted under optimal for corresponding enzyme preparations values of temperature and pH on the standard (casein on Gammersten) and specific protein substrates , which were used as native collagen protein preparation obtained from the cattle Achilles tendon and modified collagen proteins preparation obtained from splits of cattle by liquid chemical treatments [11] , the total fraction of meat water-soluble proteins and myofibrillar proteins (for example, actomyosin complex) . The temperature and pH corresponded to the middle of the interval of acid and thermal optimum of preparation effects given in Table 1.

For the water-soluble meat protein fraction obtaining distilled water mass with the ratio of 1:5 by volume was added to the weighed portion of finely ground beef muscle tissue (10 g) purified from the connective tissue, and extraction was carried out in an ice bath with continuous stirring for 30 min. After a time the extract was separated by centrifugation at 83 c^{-1} for 5 minutes followed by decantation. The protein concentration in the extract was determined by color reaction photocolorimetry with biuret reagent according to the method [12]. Calibration graph in determining the protein was built by serum albumin (M = 75000 Да). 1 micromol equiv. the amount of protein was taken giving a color reaction with biuret reagent optical density equal to the optical density of the solution of serum albumin at a concentration of 1 micromol/dm^3.

Actomyosin complex proteins were extracted by Weber's solution (1:5) after extraction of water-soluble fraction proteins of muscle in the conditions similar to extraction of the previous fraction. Protein concentration in extract was determined similar to water-soluble fraction which for receiving a number of experimental points was changed in the range of 0,1 K_m - 10 K_m by the corresponding dilution by the distilled water.

For carrying out the experiment 5 cm^3 of the protein fraction were added as it was described above, 1 cm^3 of solution of enzyme preparation. The concentration of the protein fractions and enzyme preparation selected so that reactionary mixture will have predetermined ratio of enzyme / protein (PA LU / g protein). The mixture has been incubated at 37°C for 3 hours. Protein concentration in hydrolyzates was determined by color reaction with a biuretovy reagent [12] at 550 nanometers. The reactionary non-incubated mixture was used for control. The calibration schedule was built on serum albumin.

The native and modified collagen was used in the form of water dispersion with a mass fraction of solids 25%. The molecular mass of collagen was accepted 300000 Da. The degree of hydrolysis of the native and modified collagen was after drying the solid residue of hydrolyzate up to the constant weight at 40°C.

RESULTS AND DISCUSSION

Indicator of enzyme preparation specificity is value of Michaelis-Menten's constant representing substratum concentration at which the given enzyme provides the reaction speed equal to a half of maximum possible in these conditions [13]. The kinetics analysis of the enzyme reactions is based on the Michaelis-

ISSN: 0975-8585

Menten equation (1) which allows to determine Michaelis 's (K_m) constant and the maximum reaction speed (V_{max}):

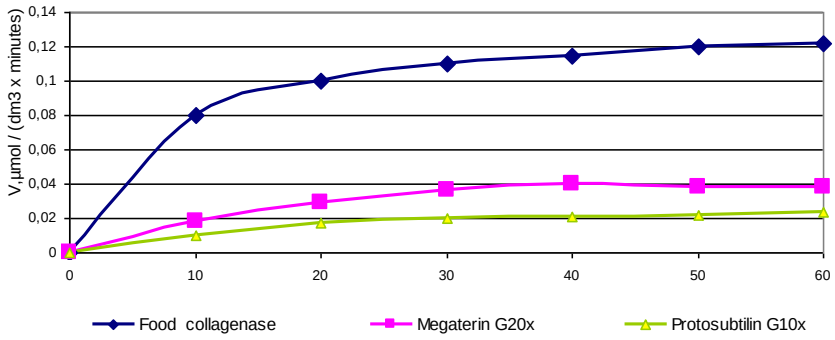
$$V_0 = \frac{V_{\max} \cdot [S]}{K_m + [S]}, \qquad (1)$$

where V_0 – the initial reaction speed in substratum concentration S, V_{max} – maximum reaction speed, K_m – Michaelis 's constant for the given composition “enzyme-substratum”.

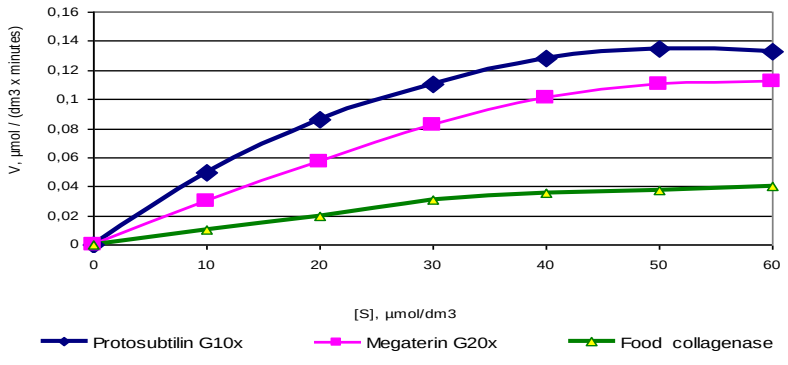
In V_{max} and K_m calculation we used linear transformation of the Michaelis-Menten equation by Lineweaver-Burk's method:

$$\frac{1}{V_0} = \frac{K_M}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}}, \qquad (2)$$

The examples of the received kinetic curves on accumulation of protein hydrolysis products belonging to the actomyosin complex of the muscle tissue and collagen proteins extracted from the tendons of cattle, are shown in Fig. 1.



a



b

Figure 1: The rate of accumulation of the animal protein hydrolysis products by the enzyme preparations: a - actomyosin complex protein; b - native collagen.

Statistical processing and linearization of experimental data in the coordinates of Lineweaver-Burk allowed to determine the kinetic constants of enzymatic catalysis of the globular and having a strengthened structure fibrous proteins (Table 2). It is of interest the comparative evaluation with the similar data for a number of other combinations of enzyme - substrate, including data obtained by other authors (A.A. Donets, 2002; V.Ya. Ponomarev, 2002).

Table 2: Michaelis 's constant (K_m, mol/dm^3) for various combinations of enzyme – substrate.

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Substrate	Food collagenase	Megaterin G10x	Protosubtilin G10x	Papain	Trypsin
Casein	$5,0\times10^{-5}$	$2,0\times10^{-5}$	$2,0\times10^{-5}$	$6,5\times10^{-5}$	$6,8\times10^{-5}$
Collagen (native)	$6,0\times10^{-6}$	$4,0\times10^{-5}$	$5,0\times10^{-5}$	$1,2\times10^{-2}$	$3,3\times10^{-3}$
Collagen (modified)	$2,0\times10^{-2}$	$5,5\times10^{-5}$	$2,3\times10^{-2}$	$1,5\times10^{-3}$	$3,3\times10^{-3}$
Meat protein water-soluble fraction [14]	$1,9\times10^{-5}$	$3,0\times10^{-5}$	$9,3\times10^{-7}$	$6,2\times10^{-7}$	$2,2\times10^{-6}$
Actomyosin complex [15]	$1,5\times10^{-5}$	$1,9\times10^{-5}$	$5,0\times10^{-6}$	$2,3\times10^{-6}$	$9,2\times10^{-5}$

* According to the data of A.A. Donets, 2002; V.Ya. Ponomarev, 2002

Unlike other enzymes collagenase shows minimum affinity ($1/K_m$) to the water-soluble protein fraction of meat proteins, under the action of salt-soluble fraction collagenase is close to trypsin and shows a pronounced affinity to native collagen, differing by the maximum rate of hydrolysis of this substrate. It is necessary to notice that papain, protosubtilin and trypsin aren't probably true collagenases, i. e. they don't affect native collagen [13]. The hydrolysis act in this case is probably caused by a thermal denaturation of collagen in the reaction medium (partially denatured collagen easily gives in to hydrolysis by the majority of known proteases), by which the process of its extraction from animal tissues is accompanied.

Equally important criterion for evaluating a substrate specificity of the enzyme is the degree of hydrolysis of the substrate. Dependence of the degree of hydrolysis on the concentration of the enzyme preparation allows to evaluate indirectly the location of proteolysis reaction equilibrium point (since the reaction is reversible). High values of the degrees of hydrolysis at low enzyme concentrations indicate a high affinity of the enzyme to the appropriate substrate.

The results of studies have confirmed that collagenase has the highest affinity to the native collagen. Thus, during the hydrolysis of water- and salt-soluble meat protein fractions the maximum obtainable hydrolysis degree was 65% (at a dosage of 10-11 units enzyme preparation. PA/g.protein) and 55% (at a dosage of 14-15 units. PA/g.protein) respectively, while, for example, these values for papain, were 92% (5.5-6.5 units PA/g.protein) and 80% (7-8 units PA/g.protein) respectively. The degree of hydrolysis of the native collagen by collagenase was not less than 85% at the preparation dosage of 4-5unit PA/g. protein; megaterin G10x was 40%; protosubtilin G10x was 48%. Such indicator for papain was 25% (9-10 units PA/g. rotein).

The comparative data on the ratio of the total degrees of hydrolysis of water- and salt-soluble fraction of meat (at the maximum degree of hydrolysis of the native collagen) are shown in Fig. 2.

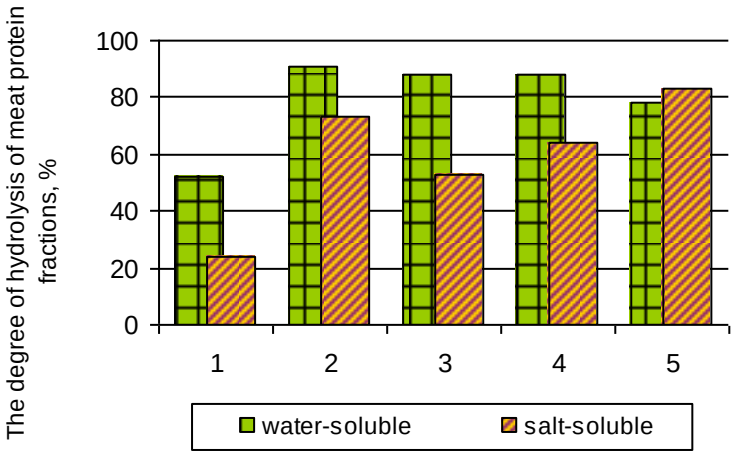

Figure 2: Meat protein fraction hydrolysis degree (1- Food collagenase, 2 – Papain, 3 – Protosubtilin G10x, 4 - Trypsin, 5 - Megaterin G20x).

According to the degree of affinity to collagenous substrates being the subject of investigation enzyme preparations are arranged in descending order: food collagenase - megaterin G10x - protosubtilin

G10x; the affinity of the preparation of collagenase to native collagen substrates exceeds affinity to the modified ones by liquid chemical treatments (splits of cattle hides).

Relatively low affinity of collagenase to water and salt-soluble proteins of meat and low maximum rate of their hydrolysis gives this preparation the advantage in differential modification of collagen proteins. Other proteolytic agents have a wide range of activity with respect to meat proteins with a predominant influence on the water- and salt-soluble fractions and therefore they can be used in processes of selective separation of the purified collagen substances of animal tissues without substantial loss of of collagen and with minimal disruption of its native structure.

The work was carried out (performed) with financial support of the Ministry of Education and Science of Russian Federation in the framework of the federal (state) task N2014/22

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