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# Isolation of *Amaranth* proteins by liquefaction with enzymes

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**ABSTRACT**

The presented work deals with possibilities in isolating amaranth protein. A biochemical procedure was chosen, consisting in employing enzymes acting specifically on protein degradation. Experiments were executed through two-level factor tests, and the influences under study were those of enzymatic hydrolysis duration (1 and 5 h) and temperature (30 and 50 $^{o}$C) on efficiency of protein hydrolysis. Amaranth flour was mixed with water in ratio 1:20, heating to desired temperature proceeded at a rate of 2 $^{o}$C.min$^{-1}$, and 0.1 % enzyme (per mass of flour) was added. Centrifugation yielded a protein hydrolysate and a solid, polysaccharide-enriched fraction.

**Key words**: Amaranth, enzyme, isolation, protein, protein hydrolysate.

## INTRODUCTION

Proteins are absolutely necessary and irreplaceable for human nourishment. Without them, build-up and reconstruction of tissues, as well as formation of proteins having a certain function in the organism (enzymes or proteins of blood plasma, nucleic acids and others) would be impossible. In case an organism has no other possibility, it even utilises proteins to cover energy requirements. Proteins have to split in several stages down to the smallest structural elements – amino acids. Only then can they be utilised. The composition and quantity of amino acids that the body itself is unable to form (essential amino acids) are the criterion by which quality of protein sources is assessed. In a global sense, the lack of proteins in nourishment is a general phenomenon. The protein deficit in industrially advanced lands is not so strong as in developing countries, but biological value of proteins is low even in highly developed countries. For this reason, value of animal proteins was disproportionately emphasised in earlier years. A majority of proteins from animal products does not wholly correspond to the requirements of human organism regarding essential amino acids content and, moreover, the intake of animal proteins in food is always connected with a high and undesirable intake of fats. Fats of protein-rich animal products are mostly represented by saturated fats exhibiting a demonstrably atherogenic influence. Vegetable proteins may be mutually combined in such manner that they result in a complete spectrum of indispensable amino acids. An optimal situation appears when man combines both vegetable and animal sources of proteins in food. A highly interesting and untraditional source of protein is amaranth grain[1,2].

Amaranth, in earlier times a decorative or utility plant, has lately been the object of keen interest especially in the food industry, as all

amaranth components (starch, proteins, fibre, fats, mineral substances, colorants, vitamins) somewhat differ from current foodstuff sources. In the last 30 years, production and consumption of amaranth have recorded significant expansion. Countries belonging to leading producers are Russia, USA, Central American countries and India. Amaranth being imported to Europe comes predominantly from these countries. From the nutritive viewpoint concerning contents of specific substances, amaranth biomass possesses an extra-ordinarily interesting composition. It contains as much as 28 % proteins per dry matter, which is up to 3 times more than in other cereals, 50 % saccharides, 6–17 % fat. An important component of amaranth fat is squalene, whose content is 8 times greater than in olive oil[3]. Amaranth biomass further contains bioflavonoids, natural pigments, minerals (in particular, Ca, Mg, Fe, K), vitamins of the B, C and K family and other minority components[4,5]. Its caloric value is 1.5 to 3 times greater than that of other cereals. Amaranth leaves, thanks to their taste properties, may be used in similar manner as another vegetable, for example, spinach. Amaranth is hence one of those precious plants that are wholly to the good – leaves as a vegetable and seed as a cereal. In order to obtain biomass from amaranth in an early stage of development, a multiple crop may be reckoned with, as amaranth harvested before start of bloom repeatedly grows up during the year. This rediscovered plant displays promising economic potential, and possibilities are being sought of how to include it in existing food products on the one hand and also to make new products from it on the other[6-7].

Amaranth grain contains more proteins than other cereals, an average 18 %, while wheat contains 10 %, rye 9 %, rice 6 % and maize 5.5 %. As much as 65 % protein in amaranth is concentrated in the sprout. Protein content varies depending on sorts of amaranth and conditions of their cultivation. Amaranth grain contains several proteins[8]. Most of the results support that albumins are the main fraction (48.9 to 65 %). Glutelin fraction is the second in abundance (22.4 to 42.3 %). Globulins represent the 13.7 to 18.1 % of the seed protein. Most data point out prolamins as the minor fraction with values between 1.0 to 3.2 %. The amount of the residue left after protein extraction is probably dependent on the buffers and the extraction time[9-11]. Amaranth protein is high-quality protein, especially rich in essential amino acids, and as it contains all essential amino acids it ranks among full-value proteins. Contents of lysine and tryptophane are comparable to those found with animal proteins[12]

Usually seed proteins are isolated using sequential solvent method. Water extraction yields albumins fraction, globulins are extracted with diluted salts, glutelins with acids or alkalis and finally ethanol gives prolamins[13]. The yield and proportions of the different protein fractions depends on the preparation method used. According to Martínez and Añón amaranth protein isolate is prepared by extraction in alkali environment. Flour is suspended in water (10 %, w/v) and pH adjusted to 8 to 11 with 2 N NaOH. The suspension is stirred for 30 min at room temperature and then centrifuged. The supernatant is adjusted to pH 5 with 2 N HCl and then centrifuged. The yield of isolated protein increased with extraction pH from approx. 5 % at pH 8 to approx. 12.5 % at pH 11 (14). Similar extraction and precipitation technique were studied to isolate amaranth protein[15].

**Objective of work**

Fractionation of amaranth flour has so far been executed by water extraction in which starch is washed out together with protein. Protein may be separated from starch only with difficulty. Amaranth protein, however, contains a high fraction of essential amino acids and can be an important component for functional foodstuffs in the field of specialised alimentary dietetics; it is thus appropriate for isolation. Within the scope of a project focused on obtaining proteins from untraditional sources, we concentrated on isolating the proteins of amaranth flour. We followed from the assumption that protein separation can be performed, beside the already mentioned graduated extraction methods, in two ways. The first consists in enzymatic degradation of polysaccharides (starch), their liquefaction into soluble glucose and enrichment of solid phase with vegetable protein. This technique was tried already earlier by applying an enzymatic preparation of $\alpha$-amylase[16]. After liquefaction, according to published results, the solid phase was enriched with protein from 15 to 30–39%, depending on conditions of

starch hydrolysis. The second method, which is the subject of this article, consists in hydrolysing proteins with proteolytic enzyme. Water-soluble protein hydrolysate is subsequently separated from the solid polysaccharide fraction by filtration. Five enzymatic preparations (proteinases) were selected to this purpose.

## MATERIAL AND METHODS

Apparatus and equipment comprised: Drier WTB Binder E/B 28 (Germany), shaft stirrer Heidolph RZR1 (Germany), electronic balance Kern 770/GS/GJ (Germany), water bath HGL W 16 (Germany), centrifuge Hettich Universal 32 (Germany), mineralisation apparatus Hach Digesdahl (U.S.A.), muffle furnace Nabertherm L 9/S 27 (Germany), polarimeter Krüss P1000 (Germany), Parnas-Wagner distillation apparatus, Soxhlet extraction apparatus.

Amaranth flour (grounded seeds of Amaranthus hypochondriacus) used in this work was supplied by the AMR Amaranth Company (Hradec Kralove, the Czech Republic); its composition is presented in Table 1.

Dry matter was determined by drying a weighed quantity of sample in glass weighing bottle at 103±2 °C for 12 hours and weighing after cooling. Inorganic solid was determined by carefully incinerating a sample of flour in a ceramic crucible over a gas burner and then by annealing at 600 °C in a muffle furnace and weighing after cooling. Total Kjeldahl nitrogen was determined by mineralising a sample of flour by boiling for 30 min (at approx. 440 °C) in sulphuric acid with added catalyst. Nitrogenous substances were thus transformed into ammonium sulphate from which ammonia was released in an alkaline environment, then steam distilled and determined by titration. Coarse proteins were determined by multiplying nitrogen content by conversion factor 5.70 [17]. Fat was extracted from the flour sample with n-hexane in a Soxhlet extraction apparatus for 4 hours. After distilling off the solvent and drying the flask containing fat for 1 hour and cooling, fat content was determined by gravimetry. Starch content was determined according to Czech standard CSN 56 0512-16 [18]. This determination is based on transforming starch into soluble starch by action of diluted HCl while warm. After clarification, soluble starch is determined by polarimetry. The method for determining fibre consists in eliminating accompanying substances from the sample by hydrolysis in an acid and alkaline medium; after a 90 min hydrolysis in 1.25 % $H_2SO_4$, the undissolved solid fraction was washed with water and hydrolysed for another 90 min in 1.25 % KOH. Non-hydrolysed residue (fibre), after washing with water and drying at 103±2 °C for 6 hours, was weighed[19].

Liquefying amaranth protein into soluble hydrolysate used 5 specific proteinases enzymes produced by deep fermentation of genetically modified microorganisms supplied by Novozymes A/S (Bagsvaerd, Denmark): Alcalase 2.5 L DX, Polarzyme 12 T, Esperase 8.0 L, Everlase 16 L EX, Savinase Ultra 16 L.

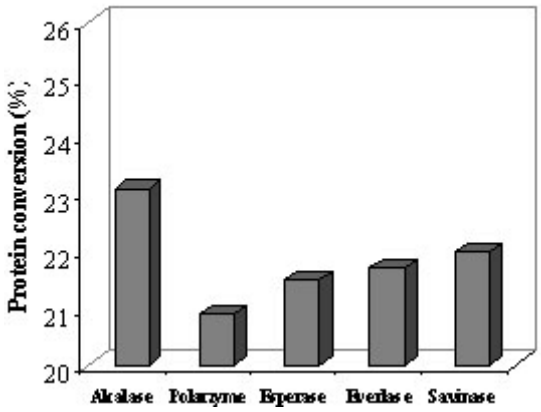
Hydrolysis of amaranth flour proteins proceeded under mild reaction conditions (temperature, atmospheric pressure, neutral pH) which were proposed and optimised to this purpose. The ratio of amaranth flour and water was 1:20. Ten grams flour was weighed to an accuracy of ±1 % into a 250-ml boiling flask, 200 ml preheated distilled water of a temperature of 22±1 °C was added and the contents were briefly stirred (approx. 20 sec). The flask containing mixture was then put into a water bath and shaft stirrer set into motion at 600 rpm, with simultaneous heating at a rate of 2 °C.min$^{-1}$. On attaining the desired temperature, 0.1 % enzyme was added (per flour dry matter). After a determined time, the mixture was centrifuged (10 min) at a rate of 6,000 rpm. The liquid phase (protein hydrolysate) and solid cake (polysaccharide basis with residually bound protein) were then analysed for dry matter content and total Kjeldahl nitrogen.

## RESULTS AND DISCUSSION

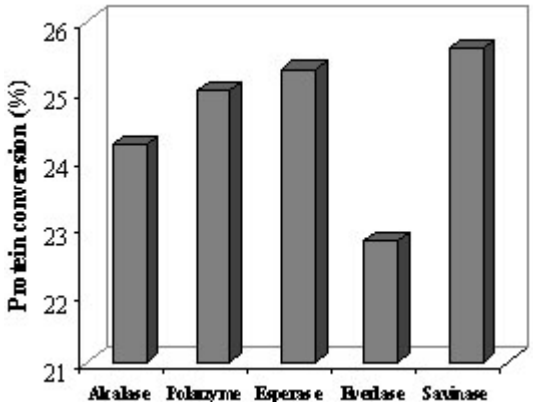
### Liquefying proteins of amaranth flour

Experiments were performed applying two-level factor tests. We investigated how duration of enzymatic hydrolysis (1 and 5 hours) and temperature (30 and 50 °C) affected protein conversion (%). Percentage of protein was calculated from the ratio of nitrogen content in hydrolysate and initial nitrogen content in amaranth

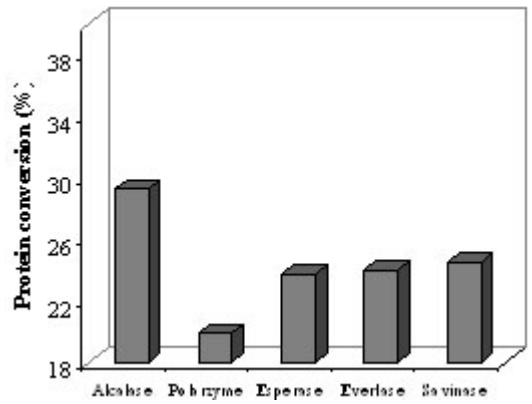
flour, and by multiplying that by conversion coefficient 5.70. Results of liquefying amaranth flour proteins are shown in Fig. 1. Hydrolysis of 1-hour duration produced changes in protein liquefying efficiency at 30 and 50 °C; by contrast, no marked differences were recorded between particular enzymes. The greatest liquefying efficiency after 1-hour hydrolysis at 30 °C was recorded with enzyme Alcalase (approx. 23%), the lowest with enzyme Polarzyme (approx. 21 %), as shown in Fig. 1-A. After hydrolysis of same duration, but at 50 °C, greatest liquefying efficiency was achieved when enzyme Savinase was employed (approx. 25.5 %), the lowest then with Everlase (approx. 23 %), as shown in Fig. 1-B. After 5-hour hydrolysis, differences in protein liquefaction at two chosen temperatures were significantly greater, as well as substantial differences recorded between particular enzymes. As shown in Fig. 1-C, at 30 °C, greatest efficiency was shown by enzyme Alcalase – approx. 29 % liquefaction. In contrast, lowest efficiency was recorded with enzyme Polarzyme – approx. 20 % liquefaction. Similarly, at 50 °C the highest liquefying efficiency for proteins was displayed by enzyme Alcalase when almost 37 % proteins were liquefied. Compared with this, enzyme Polarzyme again showed lowest liquefying efficiency – 26.5 % (Fig. 1-D).



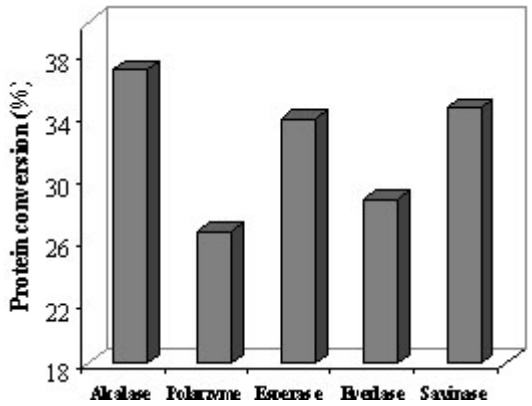

**a) hydrolysis duration 1 hour at 30 °C**




**b) hydrolysis duration 1 hour at 50 °C**




**c) hydrolysis duration 5 hour at 30 °C**




**d) hydrolysis duration 5 hour at 50 °C**

**Fig. 1: Percentage of protein conversion through application of various enzymes**

**Determining bond strength of protein to solid polysaccharide residue**

When liquefying amaranth protein by hydrolysis catalysed with proteolytic enzyme Alcalase 2.5 L DX, after centrifuging the reaction mixture there is a side product – polysaccharide residue (solid cake) containing bound residual protein. Bond strength of protein to polysaccharide may be expressed in quantitative manner by the adsorption coefficient of Langmuir's isotherm (*K*). The adsorption isotherm is generally defined as the dependence of component concentration (amaranth protein $c_A$) bound to solid phase (polysaccharide-starch) on the concentration of protein in solution ($c_0$) under equilibrium conditions. Langmuir's isotherm is quantitatively expressed by the expression as follows:

$$c_A = \frac{Ac}{1+Bc} = \frac{A\varepsilon c_0}{1+B\varepsilon c_0} \qquad ...(1)$$

In case concentrations ($c$, $c_0$) are small, product $Bc$ or $B\varepsilon c_0$ in the denominator of equation (1) may be neglected and we obtain a linear relation between $c_A$ and $C$ or $c_0$:

$$c_A = KC = K\varepsilon c_0 \qquad ...(2)$$

Linear constant $K$ was substituted here for adsorption coefficient $A$. It holds that:

$$\lim_{c\to 0} K = A \qquad ...(3)$$

When practically determining adsorption coefficient $K$, the procedure is as follows: A sample of solid phase (polysaccharide residue) of known mass and starting total protein concentration ($c_s$) is immersed in a known volume of solvent ($V_0$), clean water in our case, and the dependency of protein concentration on time is studied. On achieving a steady state, when protein concentration in the solution remains unchanged in time, it is regarded as the equilibrium concentration. A mass balance may then be performed:

$$c_s V = c_A V + c_0 V_0 + cV \qquad ...(4)$$

Applying (2) and validity

$$c = \varepsilon c_0 \qquad ...(5)$$

we may adapt and arrive at:

$$c_0 = \frac{c_s}{Na + \varepsilon(1+K)} \qquad ...(6)$$

where dimensionless consumption of solvent (water), $Na$, was introduced, defined by relationship:

$$Na = \frac{V_0}{V} \qquad ...(7)$$

In extraction by decanting, a recurrent relationship for the $(i+l)^{th}$ step in concentrating the washed-out component from the solid phase can be derived:

$$c_{0(i+1)} = \frac{\varepsilon^{i-1} c_s}{\left[Na_i + \varepsilon(1+K)\right]^i} \qquad ...(8)$$

Extraction efficiency in the $i$th step is quantitatively determined by washing-out degree ($y$), defined as the ratio of mass of washed-out component from the solid phase to total content of the same component in solid phase (in our case, % decanted residual proteins from polysaccharide residue):

$$y_i = \frac{c_{0i} V_{0i}}{c_s V} = \frac{c_{0i}}{c_s} Na \qquad ...(9)$$

Total efficiency is then determined by the sum of efficiencies in every decanting step:

$$y_n = \sum_{i=1}^{n} y_i = \frac{1}{c_s} \sum_{i=1}^{n} \frac{c_{0i}}{Na_i} \qquad ...(10)$$

Assuming the dimensionless consumption

of solvent is the same in every *i*th step *($Na_1 = Na_2 = Na_i$)* and applying (8) and (9), the final relationship determining total efficiency of multiple extractions (decanting) may be derived:

$$y_n = 1 - \left[\frac{\varepsilon(1+K)}{Na_i + \varepsilon(1+K)}\right]^n \quad ...(11)$$

From there it follows that *K* is:

$$K = \frac{(1-y)^{\frac{1}{n}}(Na_i + \varepsilon) - \varepsilon}{\varepsilon\left[1-(1-y)^{\frac{1}{n}}\right]} \quad ..(12)$$

During actual determination of adsorption coefficient (*K*), we tested 4 dimensionless solvent consumptions *(Na)*: 5, 10, 20, 100. Wet cake (dry mass 8.8 g) remaining after enzymatic hydrolysis of amaranth flour proteins was dosed. Specified dosage: with Na=5, 44 g water was dosed per wet cake; with Na=10 it was 88 g water, with Na=20 it was 176 g water and with Na=100 it was 880 g dosed water. One and four washing cycles were examined, one washing cycle lasting 20 or 60 min. Work proceeded at laboratory temperature (22±1 °C) under stirring with a shaft stirrer at 2,000 rpm in glass beakers of volumes as follows: 150 ml (Na=5), 300 ml (Na=10), 600 ml (Na=20) and 3,000 ml (Na=100). After a determined time, the mixture was centrifuged (10 min) at 6,000 rpm. The liquid phase (decanted water-soluble proteins) and remaining

**Table 1: Composition of amaranth flour**

| Parameter | Value (%) |
|---|---|
| Dry matter | 86.9 |
| Inorganic solids in dry matter | 3.6 |
| Total Kjeldahl nitrogen in dry matter | 2.8 |
| Coarse proteins (nitrogen x 5.70) in dry matter | 16.1 |
| Fat in dry matter | 9.8 |
| Starch in dry matter | 65.8 |
| Fibre in dry matter | 4.9 |

**Table 2: Decanting extraction of residual proteins from polysaccharide residue**

| Na | n | y (%) | | y | | K | |
|---|---|---|---|---|---|---|---|
| | | *Duration of one washing cycle* | | | | | |
| | | 20 min | 60 min | 20 min | 60 min | 20 min | 60 min |
| 5 | 1 | 7.14 | 10.29 | 0.0714 | 0.1029 | 131 | 86 |
| | 4 | 15.73 | 18.45 | 0.1573 | 0.1845 | 227 | 189 |
| 10 | 1 | 10.03 | 15.15 | 0.1003 | 0.1515 | 178 | 111 |
| | 4 | 19.31 | 21.71 | 0.1931 | 0.2171 | 362 | 316 |
| 20 | 1 | 12.37 | 15.26 | 0.1237 | 0.1526 | 282 | 221 |
| | 4 | 21.39 | 22.13 | 0.2139 | 0.2213 | 644 | 619 |
| 100 | 1 | 31.52 | 34.67 | 0.3152 | 0.3467 | 434 | 376 |
| | 4 | 39.41 | 40.80 | 0.3941 | 0.4080 | 1497 | 1428 |
| | | | | | Mean value of K | 469 | 418 |

Legend to Table 2: Nadimensionless consumption of solvent

n number of washing cycles y (%)% decanted residual proteins from polysaccharide residue

y washing degree K adsorption coefficient

solid fraction (polysaccharide basis with residual bound protein) was analysed for dry matter content and total Kjeldahl nitrogen. Results of decanting extraction of proteins from the polysaccharide residue remaining after enzymatic hydrolysis of amaranth flour proteins are indicated in Table 2.

Determined values of adsorption coefficient *(K)* exhibit relatively large scatter, their mean values are quite high: K=469 with a 20-min washing cycle and K=418 with a 60-min washing cycle. High levels of adsorption coefficient prove the considerable strength bonding protein to polysaccharide basis; hence, decanting extraction is of low efficiency and economically disputable.

## CONCLUSIONS

Our aim was to develop a technology for isolating amaranth protein. The selected method was a biochemical procedure consisting in employing enzymes acting specifically on protein degradation. Five enzymatic preparations (proteinases) were selected to this purpose. Water-soluble low-molecular protein hydrolysate was separated from solid polysaccharide fraction (starch) by centrifugation. From results it is obvious that the highest efficiency in liquefying amaranth flour proteins was exhibited by enzyme Alcalase. Five-hour hydrolysis at 50 °C produced 37 % protein liquefaction. The adsorption coefficient (*K*) expressing the strength bonding protein to polysaccharide remnant is quite high, which demonstrates considerable strength of this bond. Decanting extraction of proteins is of low efficiency and economically doubtful. Composition, properties, past, present and future potential applications of particular amaranth components demonstrates the food potential of this crop. Nevertheless, problems concerning commercialisation of amaranth still last, particularly due to insufficient experimental data. In agriculture, for example, it is necessary to study more closely specific soil requirements of amaranth nutrients, how to increase harvests, amaranth composition in various phases of growth, etc, as well as the choice of amaranth variants for the mild climate. In the food industry, research and development works should focus on storage properties, functionality of cereal amaranth and amaranth for protein concentrates. Furthermore, a quality deterring commercialisation is the small size of amaranth grains. For this reason, scientific teams are concerned with the search for variants yielding bigger grains or, on the other hand, adapting handling technologies to small grains. The chief challenge for research and development comprises incorporating amaranth in standing foodstuff recipes, modifying their functional and nutritive quality, creating completely new products from corn and from amaranth. It further comprises evaluating quality of amaranth-based foodstuffs and effects of particular components on the health of man, evaluating and determining the most suitable variant as feed for livestock. Not last, elaborating procedures to isolate particular components of amaranth through fractionation, and to identify their physiological activity. And for use as food additives, producing modified amaranth protein isolates with improved functional properties.

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