

Characterization of Yeast Protein Enzymatic Hydrolysis and Autolysis in *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*

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Received: 10 January 2015

Accepted: 29 April 2015

ABSTRACT: Protein recovery under sonication treatment and autolysis, also protein hydrolysis progress during enzymatic hydrolysis (using trypsin and chymotrypsin) and autolysis (using endogenous enzymes) were investigated in *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*. Crude protein content of dried yeast cells were 53.22% and 45.6% for *S.cerevisiae* and *K.marxianus*, respectively. After 96 hrs of autolysis in the presence of ethyl acetate, protein recovery reached 59.74% and 77.18% for *S. cerevisiae* and *K. marxianus*, respectively and it was 53.66% and 55.17% for sonication treatment. The yeast protein solution obtained by sonication was hydrolyzed by trypsin and chymotrypsin. Autolysis, produced hydrolysates with higher degree of hydrolysis (DH) values as compared to the enzymatic hydrolysis. After 96 hours autolysis, DH increased to 48.75% and 39.51% for *S. cerevisiae* and *K. marxianus* respectively. Chymotrypsin was significantly more effective on *K. marxianus* protein with the DH value of 21.59%, but the effect of trypsin was the same in the two yeast strains (DH value of 18.5%). It can be concluded that the autolysis process is significantly more effective than enzymatic hydrolysis by producing smaller peptides.

Keywords: Autolysis, Degree of Hydrolysis, Hydrolysis, *K. marxianus*, *S. cerevisiae*.

Introduction

Investigations of protein hydrolysis, active fractions and their functional properties in food and feed industries have resulted in the development and design of new food products. Protein hydrolysates have been used in food supplements, infant foods, sports nutrition products, and functional foods as well as for the nutritional management of consumers with digestive problems (Rutherford, 2010).

Protein hydrolysates consist of complex mixtures of free amino acids and peptides with various chain lengths. It is necessary to characterize protein hydrolysates before they can be used as a new food product. It has been shown that protein hydrolysates rich in low molecular weight peptides, especially di- and tripeptides, with minimum free amino acids, have high nutritional value. Functional properties of protein hydrolysates such as solubility, water binding, foaming and emulsification are related to molecular size distribution. Recently, protein

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hydrolysates have also been considered as a potent source of bioactive peptides, with chain lengths between 2-20 amino acids. They exhibit antimicrobial, antioxidative, hypocholesterolaemic, immunomodulatory and opioid activities as well as blood pressure-lowering effects (ACE inhibitory) and enhancement of mineral absorption (Silvestre, 1997; Rutherford, 2010).

The degree of hydrolysis (DH) is defined as the number of broken peptide bonds and is a reflection of the average size of peptides produced (Rutherford 2010; Klompong *et al.*, 2012). Different methods are used to evaluate the degree of hydrolysis that are based on three essential principles; the determination of the amount of nitrogen released, soluble protein content in the presence of a precipitation agent (e.g., TCA) and free α -amino groups and titration of the released protons (Silvestre, 1997).

Yeast hydrolysates are used as nutritional supplements, food additives and flavoring agents (Bekatorou *et al.*, 2006). It is usually produced from baker's or spent brewer's yeast by autolysis. During autolysis, the effects of autolysis enzymes including proteases, glucanases and nucleases lead to the hydrolysis of intracellular compounds (Verduyn *et al.*, 1999). On the other hand yeast protein might be considered as a substrate for proteolytic enzymes such as trypsin and chymotrypsin. Trypsin hydrolyses peptide bonds at lysyl and arginyl residues that furnish the carbonyl part of the peptide bond, and chymotrypsin hydrolyses peptide bond only when tyrosine, phenylalanine or tryptophan residues provide the carbonyl group of the peptide bond. *S. cerevisiae* is an important yeast that is used to produce single cell protein (SCP). Different reports showed that the percentages of lysin, arginine, phenylalanine, tyrosin and tryptophan are 6.19%, 4.07%, 3.29%, 2.95% and 1.08% respectively for *S. cerevisiae* whereas the percentages of the same amino acids in the

SCP produced from *K. marxianus* are 6.51%, 4.20%, 3.71%, 3.15% and 0.98% (John, 2002; Qverland *et al.*, 2013). These differences might be the result of the production of protein hydrolysates with different characteristics.

To our knowledge there is no report comparing the properties of yeast hydrolysates obtained from enzymatic hydrolysis and autolysis. The aim of this research was to compare the hydrolysis and autolysis in the two yeast strains based on measuring DH values. Sonication treatment and autolysis processes were used for protein recovery from *S. cerevisiae* and *K. marxianus* cells. Sonication treated samples were considered as a substrate for the two different proteolytic enzymes (trypsin and chymotrypsin). Characteristics of the protein hydrolysate were compared with autolysis products based on measuring DH. The effect of intrinsic yeast cell enzymes and the combination effect of trypsin and chymotrypsin was also studied. In this paper, enzymes released from yeast cells during autolysis or sonication treatments are called intrinsic enzymes.

Materials and Methods

- Materials

Kluyveromyces marxianus (PTCC 5195) and *Saccharomyces cerevisiae* (PTCC 5269) were obtained from Persian-type culture collection (PTCC) of the Iranian Research Organization for Science and Technology (IROST). Chymotrypsin (EC 3.4.21.1, containing 55 units/mg solid), Trypsin (EC3.4.21.4; containing 1300 BAEE units/mg solid) and ortho-phthaldialdehyde (OPA) were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). All the chemicals were of analytical grade.

One unit of chymotrypsin activity is defined as the amount that hydrolyses 1.0 μ mol of BTEE per min at pH of 7.8 at 25°C, and one BAEE unit of trypsin activity is defined as the amount that produces a ΔA_{253}

of 0.001 per min at pH of 7.6 at 25° C using BAEE as a substrate.

- *Culture conditions*

K. marxianus and *S.cerevisiae* were grown in yeast mold (YM) culture. The temperature was controlled at 28°C in a shaking incubator (IRC-1-U, Clim-O-shake, Switzerland) at 150 rpm. Cells were harvested in the middle of logarithmic phase by centrifugation at 3000×g for 10 min. The yeast pellet was washed with distilled water three times and stored at -20°C.

- *Total protein assay:*

Total protein content of yeast cells was measured by the Kjeldahl method via multiplication of total nitrogen by 6.25 (AOAC, 2000).

- *Protein recovery by sonication*

Suspensions of 2.5% of dry yeast cells in 50 ml of distilled water were sonicated in a sonicator (Part NO. S-4000) at a fixed power of 600 W, frequency of 20 kHz and amplitude of 50%. Total cycle time for ultrasonic treatment was 10 min. The pulse duration and pulse intervals were each one minute. The jar was immersed in an ice-water bath to avoid a temperature increase during sonication. Cell debris and particles were removed by centrifugation at 11500×g for 10 min. Protein content of supernatant was determined by Lowry's method, modified by Hartree. Bovine serum albumin (BSA) was used as the protein standard (Lowry et al., 1951; Hartree, 1972). Samples were stored at -20°C for further analysis.

- *Preparation of yeast hydrolysate samples*

A final concentration of 4 mg/ml protein solution in 50 mM phosphate buffer obtained after centrifugation was heated at 37°C with continuous shaking using shaking water bath (120RPM). Trypsin or chymotrypsin were added in appropriate quantities to attain the enzyme, substrate

ratio of 1:10 (W/W). The reaction time was 5 hrs. The pH and temperature values were adjusted to the optimal conditions of the enzymes (pH=7.8 and 37°C). The effects of hydrolytic intrinsic yeast cell enzymes on the progress of hydrolysis were also studied. Designation of the groups studied in this research are as the followings,

- a- 5 hrs of hydrolysis with intrinsic yeast cell enzymes as the control sample.
- b- 5 hrs of hydrolysis with individual activity of trypsin and chymotrypsin enzymes (intrinsic yeast cell enzymes were thermally inactivated).
- c- 5 hrs of hydrolysis with individual activity of trypsin and chymotrypsin enzymes (intrinsic yeast cell enzymes were not inactivated).
- d- 5 hrs of hydrolysis with combination of trypsin and chymotrypsin in sequence after the inactivation of the first enzyme by heating at 90°C for 10 min.

- *Preparation of yeast autolysate samples*

For the autolysis experiment, cells were collected in the stationary growth phase by centrifuging at 3000×g at 15 min, and washed with distilled water. 50 ml of 2.5% dry yeast cells in distilled water was incubated at 52°C and pH of 5 (Běchalová & Beran, 1986) with agitation at 120 rpm for up to 96 hrs. To one sample ethyl acetate was added to a final concentration of 1.5% (V/V) (Conway et al., 2001; Wang et al., 2003) as the autolysis inducer and the other sample was prepared without any inducer. Samples were withdrawn every 24 hrs, heated at 85°C for 15 min to terminate the enzyme activity (Tanguler & Erten, 2008), and cooled to 4°C. Cell debris was removed by centrifugation at 11500×g for 10 min at 4°C. Protein content of the supernatant during autolysis process was determined by Lowry's method, modified by Hartree using BSA as the protein standard (Lowry et al., 1951; Hartree, 1972).

- Evaluation of the degree of hydrolysis

The extent of hydrolysis was determined using two different methods. First, the degree of hydrolysis was assayed directly by measuring cleaved peptides bonds using O-phthaldialdehyde (OPA) spectrophotometric assay and second by the determination of soluble protein in trichloroacetic acid (TCA) (Chae *et al.*, 2001; Rutherford, 2010) as described below:

a. OPA- Spectrophotometric assay

This assay is used for the reaction of free α -amino nitrogen with O-phthaldialdehyde (Salami *et al.*, 2008; Jamdar *et al.*, 2010; Sun *et al.*, 2011). A fresh OPA solution was prepared daily as follows: 25 ml of 100 mM sodium tetrahydroborate, 2.5 ml of 20% SDS (W/V), 40 mg of OPA (dissolved in 1 ml methanol) and 100 μ l of β -mercaptoethanol were mixed and adjusted to a final volume of 50 ml with distilled water. To assay proteolysis with yeast protein as substrate, 10-50 μ l aliquots containing 5-100 μ g protein were added directly to 1 ml of OPA reagent. The solution was mixed briefly and incubated for 2 min at room temperature. Subsequently the absorbance was read at 340 nm using a spectrophotometer (Jenway, Model, 6315). L-leucin (0-4 mg/ml) was used to construct a standard curve and free amino groups were calculated using the standard curve. DH was calculated using the following formula:

$$DH = \frac{L1 - L0}{Lmax - L0}$$

L1: Number of free amino groups released after hydrolysis.

L0: Number of free amino groups in the original yeast extract.

Lmax: Total number of free amino groups in the original yeast extract obtained after acid hydrolysis (6N HCl at 120°C for 24 hrs). (Jamdar *et al.*, 2010)

b. Soluble TCA-protein assay

The percentage of solubilized protein in 10% trichloroacetic acid (TCA) in relation to the protein content of the samples was measured as described previously (Hayashi *et al.*, 1987; Hoyle & Merritt, 1994; Peñas *et al.*, 2004; Harriman *et al.*, 2013). Briefly, aliquots of equal volumes of the sample and TCA solution (20%) were mixed and kept at room temperature for 30 mins, followed by centrifugation at 3000×g for 10 min. Soluble protein content of the supernatant was determined by Lowry's method, modified by Hartree (Lowry *et al.*, 1951; Hartree, 1972). Degree of hydrolysis was expressed as mg of protein soluble in TCA in relation to the total protein content according to the following formula:

$$DH(\%) = \frac{\text{Soluble protein content in 10\% TCA (mg)}}{\text{Total protein content (mg)}}$$

- Statistical analysis

In this study all the measurements were carried out twice and the results are the average of the two replications. Data are presented as mean value \pm standard deviations. The significance between mean values was determined using independent-samples T test and one-way ANOVA. Statistical analysis was performed using SPSS software, version 20. P value <0.05 was considered significant.

Results and Discussion

- Protein content of yeast cells

Cellular composition of yeast varies according to species, culture media composition, methods of cultivation and the phase of cell harvesting. In our research, crude protein contents of dried yeast cell were obtained from the total nitrogen using 6.25 as the kjeldahl nitrogen factor and these were found to be 53.22% and 45.6% for *S. cerevisiae* and *K. marxianus* respectively. The results of this experiment are in

Table 1. Protein recovery (mg/gr yeast dry weight) during autolysis period (pH=5, 52°C, 120 rpm, with and without ethyl acetate) in *S.cerevisiae* and *K.marxianus*.

Yeast	Autolysis time(hrs)	Protein(mg/gr yeast dry weight)	
		EthEyl acetate	No ethyl acetate
<i>S. cerevisiae</i>	0	0 ^a	0 ^a
	24	259.3±0.42 ^b	172.8±0 ^b
	48	294.28±0.24 ^c	216.21±1.7 ^c
	72	306.86±0 ^d	256.79±4.09 ^c
	96	316.8 ±0 ^c	282.04±0 ^c
<i>K. marxianus</i>	0	0 ^a	0 ^a
	24	272.05±0.049 ^b	83.42±0 ^b
	48	306.83±0.042 ^c	103.24±0.049 ^c
	72	316.4±0.56 ^d	123.08±0.021 ^d
	96	354.33±0.41 ^c	123.14±0 ^d

Each value is the average ± standard deviation of two replicates. Values with different letters are statistically different at P<0.05.

agreement with data published by several researchers (Orban *et al.* 1994; Chae *et al.*, 2001; Apar & Özbek, 2008) who found values of 47-57% for the protein content of yeast cells.

- Protein recovery under autolysis and sonication treatment

The results of protein extraction during the autolysis process is presented in Table 1. Protein recovery increased during the first 24 hrs (P<0.05) followed by a slight increase up to 96 hrs of autolysis (22% and 30.24% increase with ethyl acetate and 30.24% and 47.61% increase without ethyl acetate for *S. cerevisiae* and *K. marxianus* respectively). Similar results have been reported by researchers (Tanguler & Erten, 2008) who showed a quick increase in protein recovery within 24 hrs and lower increase until 96 hours.

After 96 hrs of autolysis, the amount of recovered protein reached 282.04 and 123.14 mg/g yeast dry weight (53.19% and 27.32% of total protein) for *S. cerevisiae* and

K. marxianus respectively. However, other researchers (Lukondeh *et al.*, 2003) have reported 21% and 17% protein recovery for these yeast species during 10 days of autolysis at 40°C. The difference might be due to the lower temperature of autolysis, since it is reported that at 40°C autolysis is slower than 50°C (Běchalová and Beran, 1986). Other researchers (Tanguler & Erten, 2008) reported 70% protein recovery from *S. cerevisiae* after 24 hrs of autolysis at 50°C. The results showed a significant difference (P<0.05) in the protein recovery between the two yeast strains that were confirmed by other researchers (Alexandre & Guilloux-Benatier, 2006).

The important role of organic solvents for accelerating the process of cell autolysis has been reported previously (Wang *et al.*, 2003). The effect of ethyl acetate on autolysis is due to its ability to dissolve lipids and consequently change the cell membrane permeability. An ideal concentration for autolysis of *S. cerevisiae* was reported (Wang *et al.*, 2003) when ethyl acetate was supplemented in the range of

1.5-2.4%. According to our results ethyl acetate significantly increased protein recovery ($P < 0.05$) in two yeast strains, as was previously reported for *S. cerevisiae* (Champagne *et al.*, 1999). After 96 hrs of autolysis in the presence of ethyl acetate, the amounts of recovered protein reached 316.8 and 354.33 mg/g yeast dry weight (59.74% and 77.18% of total protein) for *S. cerevisiae* and *K. marxianus* respectively. The difference in protein recovery between the two yeast strains was not significant ($P > 0.05$). The results of sonication treatment as a physical disruption method in protein recovery are presented in Table 2 (53.66% and 55.17% of total protein content for *S. cerevisiae* and *K. marxianus* respectively). During sonication treatment high shear forces induced by the collapsing cavitation bubbles leads to cell disruption and protein release. Our results showed no significant ($P = 0.122$) difference between the protein recovery of the two yeast strains. It is clear

that protein extraction during sonication treatment is related to the differences in the acoustic pressure distribution during sonication treatment influenced by different factors such as system geometry, sonication power, medium and sonication cell properties (Baldyga *et al.*, 2012). A higher degree of yeast cell disruption and protein release by increasing acoustic power and duty cycle has been reported previously (Apar & Özbek, 2008; Liu *et al.*, 2013) and protein release was found almost independent of yeast cell concentration (Apar & Özbek, 2008).

Figure 1 shows that autolysis and sonication treatment did not cause a significant difference ($P > 0.05$) in protein recovery from *S. cerevisiae*, but sonication is more effective than autolysis. Autolysis in the presence of ethyl acetate makes it a more effective treatment for protein recovery in both yeast strains ($P < 0.05$).

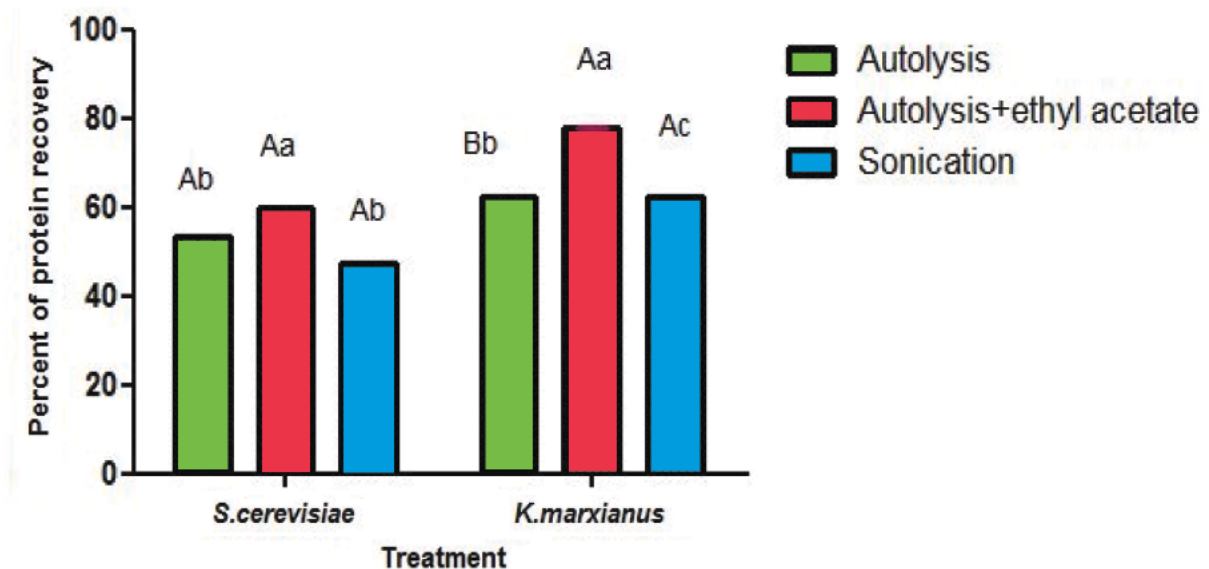


Fig. 1. Comparing percent of protein recovery in autolysis process (pH=5, 52°C, 120 rpm, 96 hours, with and without ethyl acetate) and sonication treatment (fixed power of 600 W and amplitude of 50, 10 min) in *S. cerevisiae* and *K. marxianus*. Values with different small letters for comparing treatments in every yeast strain and capital letters for comparing two yeast strain are significantly different at $P < 0.05$.

Table 2. Protein recovery of sonication treated (fixed power of 600 W, amplitude of 50% and 10 min.) yeast cells.

Yeast	Protein		
	Percent of total protein	mg/ml (extracted from 2.5% suspension of dry cell in distilled water)	mg/gr yeast dry weight
<i>S.cerevisiae</i>	53.66	6.2	251
<i>K.marxianus</i>	55.17	7.1	285

Each value is the average \pm standard deviation of two replicates.

Table 3. Progress of hydrolysis (OPA method) in yeast extract (*K.marxianus*) under controlled conditions (pH=7.8, 37°C, 120 rpm)

Time(hrs)	Degree of hydrolysis (%)				
	Experiment a	Experiment b		Experiment c	
	Control	Trypsin	Chymotrypsin	Trypsin+intrinsic enzymes	Chymotrypsin+intrinsic enzymes
0	11.67 \pm 0.091 ^a	11.75 \pm 0.023 ^a	11.02 \pm 0.311 ^a	11.05 \pm 0.07 ^a	11.9 \pm 0.15 ^a
0.5	11.56 \pm 0.063 ^a	16.165 \pm 0.035 ^b	19.97 \pm 1.18 ^{ab}	13.57 \pm 0.304 ^b	16.05 \pm 0.19 ^b
1	12.67 \pm 0.035 ^b	16.98 \pm 0.042 ^c	19.895 \pm 0.19 ^{ab}	16.58 \pm 0.00 ^c	18.75 \pm 0.56 ^b
1.5	13.79 \pm 0.00 ^c	17.37 \pm 0.00 ^{cd}	20.37 \pm 0.00 ^b	16.99 \pm 0.12 ^c	21.6 \pm 0.028 ^c
2	14.08 \pm 0.00 ^d	18.28 \pm 0.056 ^d	21.28 \pm 0.056 ^{bc}	19.91 \pm 0.014 ^{cd}	22.47 \pm 0.11 ^c
2.5	14.73 \pm 0.00 ^d	18.36 \pm 0.068 ^d	21.36 \pm 0.063 ^{bc}	20.16 \pm 0.007 ^d	23.63 \pm 0.43 ^{cd}
3	15.82 \pm 0.00 ^d	18.41 \pm 0 ^d	21.44 \pm 0.049 ^{bc}	20.43 \pm 0.3 ^d	24.55 \pm 0.00 ^d
3.5	15.73 \pm 0.00 ^e	18.41 \pm 0.007 ^d	21.49 \pm 0.00 ^{bc}	20.78 \pm 0.07 ^d	25.96 \pm 0.33 ^d
4	15.86 \pm 0.00 ^f	18.51 \pm 0.00 ^d	21.51 \pm 0.01 ^c	20.56 \pm 0.091 ^d	26.08 \pm 0.035 ^d
4.5	15.94 \pm 0.021 ^f	18.52 \pm 0.021 ^d	21.55 \pm 0.056 ^c	20.16 \pm 0.00 ^d	26.11 \pm 0.19 ^d
5	15.91 \pm 0.169 ^f	18.52 \pm 0.021 ^d	21.59 \pm 0.00 ^c	21.39 \pm 0.007 ^d	26.18 \pm 0.098 ^d

Each value is the average \pm standard deviation of two replicates. In each column, different small letters indicate significant at P<0.05). Treatments a, b and C describe respectively, 5 hours of hydrolysis with intrinsic yeast cell enzymes as control sample, 5 hours of hydrolysis with individual activity of trypsin and chymotrypsin enzymes (intrinsic yeast cell enzymes were thermally inactivated) and 5 hours of hydrolysis with individual activity of trypsin and chymotrypsin enzymes (intrinsic yeast cell enzymes were not inactivated).

- Characterization of yeast autolysate

Figure 2 shows the progress of autolysis (in the presence of ethyl acetate) based on measuring the degree of hydrolysis over 96 hours using TCA-soluble protein content and OPA assay. During the first 24 hrs the rate of autolysis is high in both strains, and continues to decrease thereafter. Finally a DH of 77.4%, 48.75% (for *S. cerevisiae*) and 55.86%, 39.51% (for *K. marxianus*) is

measured for TCA soluble protein and OPA methods, respectively. During autolysis the amounts of extracted protein increase significantly such that the increase in DH might be attributed to the protein content of the autolysate and to proteinases and peptidases that have been released into the autolysates that continue their action during the autolysis period, as was previously reported (Lukondeh *et al.*, 2003). A

significantly higher degree of protein hydrolysis ($P<0.05$) was found in *S. cerevisiae* as compared to *K. marxianus* during autolysis. According to other researches (Hunter & Asenjo, 1986; Amrane & Prigent, 1996) the critical step of the autolytic process in *S. cerevisiae* and *K. marxianus* is biosynthesis of lytic enzymes during cell growth rather than enzymatic activity during autolysis.

- Characterization of yeast protein hydrolysate

The results of enzymatic hydrolysis in the three experiments are presented in Tables 3 and 4. By considering the progress of hydrolysis up to 5 hours (Tables 3 and 4) it can be concluded that in all treatments and for two yeast strains, essentially most effective hydrolysis occurs during the first hours and continues at a lower speed thereafter. Thus after 5 hours of trypsin and chymotrypsin activity, DH values of 18.51%

and 17.87% for *S. cerevisiae* and 18.52% and 21.59% for *K. marxianus* were obtained using OPA assay. The results of Table 5 indicate that trypsin and chymotrypsin hydrolysis, lead to significant increase ($P<0.05$) in DH values as compared to the control sample. Although there is no significant difference ($P>0.05$) in trypsin activity between the two yeast strains, chymotrypsin was more effective on the *K. marxianus* protein. This might be contributed to the greater number of potential present hydrolytic sites targeted by chymotrypsin (John, 2002).

Simultaneous activity of intrinsic yeast cell enzymes during trypsin and chymotrypsin hydrolysis (experiment C) caused a significant ($P<0.05$) increase in the intensity of hydrolysis. Using a combination of trypsin and chymotrypsin did not significantly ($P>0.05$) increase the degree of hydrolysis as compared to the hydrolysis by individual enzymes (Table 2).

Table 4. Progress of hydrolysis (OPA method) in yeast extract (*S.cerevisiae*) under controlled conditions (pH=7.8, 37°C, 120 rpm)

Time (hrs)	Degree of hydrolysis (%)				
	Experiment a	Experiment b		Experiment c	
	Control	Trypsin	Chymotrypsin	Trypsin+intrinsic enzymes	Chymotrypsin+intrinsic enzymes
0	10±0 ^a	10.59±0.24 ^a	11.77±0 ^a	10±0 ^a	10±0 ^a
0.5	11.27±0.035 ^b	11.6±0 ^a	12.04±0 ^b	11.42±0.3 ^a	13.095±0.7 ^a
1	13.81±0.021 ^c	15.77±0.14 ^b	16.33±0.042 ^c	20.54±1.25 ^b	20.005±0.75 ^b
1.5	13.98±0.07 ^c	16.49±0.11 ^b	16.41±0.148 ^c	21.38±0.86 ^b	22.07±0.93 ^b
2	13.42±0.035 ^c	16.63±0.021 ^b	16.36±0.403 ^c	22.73±0.88 ^b	25.35±0.46 ^{bc}
2.5	13.84±0.00 ^c	16.87±0.07 ^b	17.18±0.019 ^d	23.645±0.162 ^b	25.47±0.5 ^{bc}
3	13.85±0.23 ^c	18.36±0.17 ^c	17.50±0.01 ^d	24.95±0.021 ^b	31.39±0.056 ^c
3.5	13.07±0.098 ^c	18.5±0.007 ^c	17.66±0.24 ^d	25.01±0.098 ^b	30.88±0.46 ^c
4	13.1±0.33 ^c	18.51±0.00 ^c	17.84±0.03 ^d	28.11±0.162 ^c	31.49±0 ^c
4.5	12.2±0.268 ^c	18.51±0.00 ^c	17.84±0.00 ^d	28.06±0.091 ^c	32.15±0.268 ^c
5	13.01±0.28 ^c	18.51±0.00 ^c	17.87±0.00 ^d	27.99±0.007 ^c	32.505±0.1 ^c

Each value is the average ± standard deviation of two replicates. In each column, different small letters indicate significant at $P<0.05$. Treatments a, b and C describe respectively, 5 hours of hydrolysis with intrinsic yeast cell enzymes as control sample, 5 hours of hydrolysis with individual activity of trypsin and chymotrypsin enzymes (intrinsic yeast cell enzymes were thermally inactivated) and 5 hours of hydrolysis with individual activity of trypsin and chymotrypsin enzymes (intrinsic yeast cell enzymes were not inactivated).

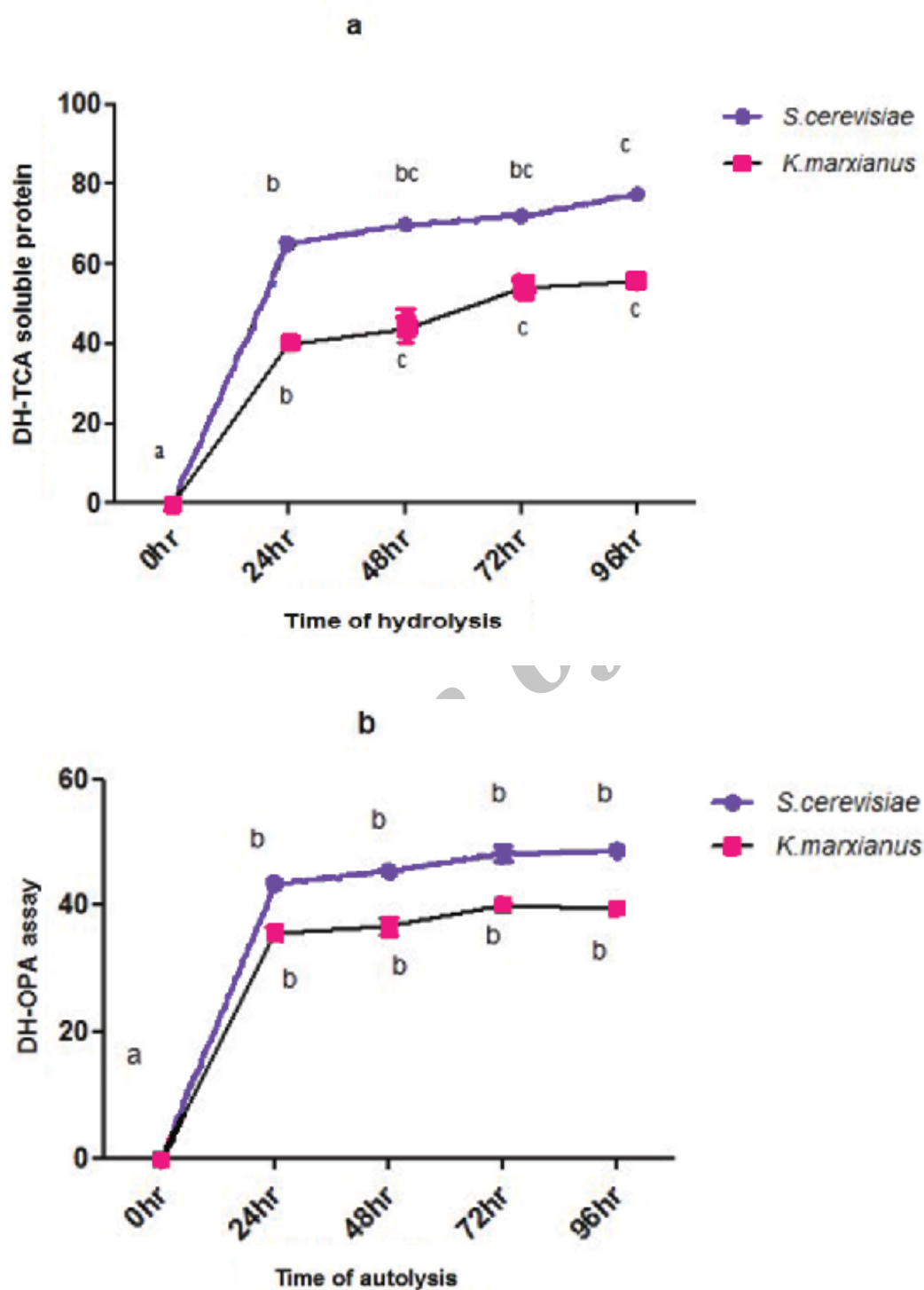


Fig. 2. Progress of autolysis (a) TCA soluble protein method and (b) OPA method in yeast (*S.cerevisiae* and *K.marxianus*) suspension under controlled conditions (pH=5, 52°C, 120 rpm, final concentration of 1.5% ethyl acetate). Values with different small letters are statistically different at $P < 0.05$.

- TCA- soluble protein assay versus OPA method

Despite extensive usage of TCA-induced protein precipitation, its molecular mechanism is not completely clear (Rajalingam *et al.*, 2009). It is suggested that TCA forces the protein to precipitate by sequestering the protein-bound water (Tanford, 1964). Other researchers (Kumar *et al.*, 1994; Xu *et al.*, 2003) have proposed that the acidic nature of TCA is the reason for conformational changes in protein structure and its precipitation. According to our results, during enzymatic hydrolysis and autolysis process (Figure 2), the DH values obtained for TCA-soluble protein assay are significantly ($P<0.05$) higher than the OPA method. This can be contributed by the partial precipitation of non-hydrolysed proteins, as some researchers (Moughan *et al.*, 1990) have shown that as much as 70% of proteins with a mass greater than 10 KD are obtained from endogenous digesta collected from the distal ileum 2 of the laboratory rats were not precipitated using TCA. Others (Rajalingam *et al.*, 2009) reported that unfolded and denatured proteins have a lower tendency to precipitate

in TCA. Furthermore, the presence of glycoproteins might cause an overestimation of DH value in the TCA-soluble protein method (Rutherford, 2010). Higher DH values were measured by TCA-soluble protein assay than the OPA method in the hydrolysis of concentrated whey protein by proteases from *Bacillus licheniformis* (Harriman *et al.*, 2013), and a discrepancy between the two methods for high pressure treated soybean whey proteins (Peñas *et al.*, 2004) was reported previously.

According to our results, TCA-soluble protein assay did not show the progress of trypsin and chymotrypsin hydrolysis. This might be explained by the low intensity of hydrolysis that leads to peptide bond breakage but not increasing peptide solubility. As has been described previously (McSweeney & Fox, 1997) there is no precipitation threshold of peptide size to solubility in TCA. Peptide solubility in TCA is also related to its hydrophobicity. Our research confirmed that TCA-soluble protein assay sensitivity depends on the size of the peptides produced, and we did not find it to be a suitable method for considering the hydrolysis process.

Table 5. Comparing different DH value after 5 hrs of hydrolysis in yeast extract of *S.cerevisiae* and *K.marxianus* in controlled conditions (pH=7.8, 37°C, 120 rpm).

Yeast	Degree of hydrolysis (%)				
	Experiment a	Experiment b		Experiment C	
	Control	Trypsin	Chymotrypsin	Trypsin+intrinsic enzymes	Chymotrypsin+intrinsic enzymes
<i>S. cerevisiae</i>	18.43±5.47 ^{Aab}	16.39±2.74 ^{Aa}	16.321±2.23 ^{Aa}	22.17±6.1 ^{Abc}	24.94±7.72 ^{Ac}
<i>K. marxianus</i>	14.34±1.67 ^{Ba}	17.38±1.97 ^{Ab}	20.135±3.029 ^{Bc}	18.32±3.3 ^{Bbc}	21.95±4.72 ^{Ac}

Values are average of DH values obtained during hydrolysis process for each treatment. In each column, different capital letters indicate significant differences ($P<0.05$). In each row, different small letters indicate significant differences ($P<0.05$). Treatments a, b and C describe respectively, 5 hours of hydrolysis with intrinsic yeast cell enzymes as control sample, 5 hours of hydrolysis with individual activity of trypsin and chymotrypsin enzymes (intrinsic yeast cell enzymes were thermally inactivated) and 5 hours of hydrolysis with individual activity of trypsin and chymotrypsin enzymes (intrinsic yeast cell enzymes were not inactivated).

Conclusion

It might be concluded that during autolysis process more DH values are obtained as compared to the enzymatic hydrolysis in both yeast strains. Therefore smaller peptides size production with different nutritional values, functional properties and bioactivity is expected. Higher intensity of autolysis was observed in *S. cerevisiae* as compared to *K. marxianus*. Although effect of trypsin was not different in two yeast strains but chymotrypsin caused higher degree of hydrolysis in *K. marxianus*.

Acknowledgment

The support of the Iranian Research Organization for Science and Technology (IROST) is gratefully acknowledged.

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