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The influence of microelements selenium and copper on the rye malt amylase activity and flour technological properties

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Abstract. The positive effect of microelement selenium (Se) on the amylase activity and technological properties of malt is well known. Copper (Cu) is an essential microelement required for the normal functioning of living organisms, plants and most microorganisms. The aim of the current research was to investigate the interaction of two microelements - copper and selenium and its influence on the rye malt and flour properties. Rye grain of 96% viability were soaked and germinated at temperature +6 ± 2 °C for 3 days, using Se (VI) containing solution (Se concentration 8.5 mg L^{-1}) or selenium with copper(II) containing solutions (Se concentration 8.5 mg L^{-1}, Cu concentrations 3 mg L^{-1}, 5 mg L^{-1}, 10 mg L^{-1}). After that sample were dried in the oven for 24 hours at temperature of + 73–108 °C. Control sample-germinating rye grain without microelements additives. Activity of amylase was determined in all experimental samples, because it characterizes the malt quality. Amylases are starch hydrolysing enzymes; more over there are known several amylases: α-amylases, β-amylases, isoamylases, etc. with different mechanisms of reaction. There different analytical methods were used for determination of α-amylase activity. The first was Ceralpha method (Megazyme test kits). The second method use complete reagents for quantitative determination of α-amylases (Phadebas Amylase Test). The third was iodometrical method. Different amounts of malt fortified with Se and Cu were added for investigation of rye flour technological properties. The falling number and the maximum viscosity were determined. The obtained results show that analysed additives of microelement copper decreases the enzyme activity. Analysed rye flour technological properties were better using malt only with selenium supplement.

Key words: selenium, copper, rye malt.

INTRODUCTION

Rye (*Secale cereale* L.) is a major crop in Russia, Poland, Germany and the Scandinavian countries, where it is the major bread grain. Rye is also used to produce crisp bread and alcohol, and it is used as animal feed (McKevith, 2004). In Latvia a popular tradition is to use this grain for baking rye bread (Gailîte et al., 2013). Substantial quantities are also used as raw material in the distilling industry. There are some specialty beers in Germany and Austria made from rye malt (Hübner et al., 2010). Rye malt is the dried product of rye germinated under controlled conditions and is widely used in the production of bread, food flavoring, as ingredient for bakery products and as

color additives in the preparation of caramel. During germination (i.e. hydrothermal treatment in ambient conditions) the biosynthetic potential of grain is exploited and a number of hydrolytic enzymes are synthesised (Kaukovirta-Norja et al., 2004). Amylases play a significant role in grain germination and it is a key quality parameter in the commercial utilization of most cereals (Muralikrishna & Nirmala 2005). In germinating grain, starch degradation is initiated by α-amylase. α-amylase produces soluble oligosaccharides from starch, which are then hydrolysed by β-amylase to liberate maltose. Finally, α-glucosidase breaks down maltose into glucose (Yamasaki, 2003).

Many methods for the assay of α-amylase are described in the literature. The simplest and most direct is Ceralpha method (Megazyme test kits), using Complete reagents for quantitative determination of α-amylases (Mccleary et al., 2002). Other determination method of α-amylase activity is Phadebas Amylase Test, but for total amylase activity iodometrical method can be used. The falling number and the maximum viscosity characterize rye flour technological properties (Buchanon & Nicholas 1980; Cauvain & Young 2009; Antoņenko et al., 2014).

Selenium (Se) and copper (Cu) is essential microelements for animals and humans, they have also been found to be beneficial to plants (Vinit-Dunand et al., 2002; Weinstein et al., 2011; Fenga et al., 2013). Trace amounts of Se can promote growth in a variety of plants and protect plants from biotic and abiotic stress. Se may activate antioxidant system by elevating glutathione peroxidase (GPX) expression under abiotic stress conditions such as ultraviolet radiation, and low temperature (Wang et al., 2012), drought, water, salinity and heavy metals (metalloids) (HMs) (Fenga et al., 2013). Besides, supply of Se protects plants from a variety of herbivores and pathogens (Wang et al., 2012). Numerous researches show the effects of Se on wheat, barley and oat sprouting activity and positive influence on biologically active substances and high vitamins concentration in germinated grain (Dūma, 2010; Antoņenko et al., 2013b) as well as the rye malt quality: content of malt extract, diastase activity, total phenols and selenium accumulation degree in rye malt (Antoņenko et al., 2014). Copper is an essential micronutrient for plants that is a component of several electron transport enzymes (Lombardi & Sebastiani 2005), it is a component of various proteins, and particularly those involved in both the photosynthetic (plastocyanin) and the respiratory (cytochrome oxidase) electron transport chains (Baro´n et al., 1995). In plants copper interacts with a wide range of physiological and biochemical processes in cells (Caspi et al., 1999). But it also induces toxicity at tissue concentrations slightly above its optimal levels (Lombardi & Sebastiani, 2005). For higher plants the tolerance limit of Cu^{2+} is 10^{-6} mol L^{-1} (Krylova & Vassiljeva, 2011).

There are many investigations about microelements metabolism and accumulation in plant, health benefits, phytoremediation and bio fortification, but information about Se and Cu influence on the rye malt amylase activity is scarce. The aim of this research was to investigate the influence of microelements selenium and copper on the rye malt amylase activity and flour technological properties.

MATERIALS AND METHODS

Plant material

The research object was rye grain (variety ´Kaupo´) from Ltd. ´Naukšēni´, harvested in 2013. Rye grain of 96% viability were soaked and germinated at

temperature 6 ± 2 °C for 3 days. Five samples of rye malt were prepared. Control sample (I) without microelement additives. The II sample contains sodium selenate (Se concentration 8.5 mg L^{-1}). Samples III, IV and V besides selenium (Se concentration 8.5 mg L^{-1}) contain copper sulphate (copper concentrations 3 mg L^{-1}, 5 mg L^{-1} and 10 mg L^{-1}). After germination grains were dried in the oven for 24 hours at temperature of 73–108 °C and ground with laboratory mill fitted with a 0.4 mm sieve. Moisture of malt samples ranged from 7.2% till 8.9%.

Determination of total amylases activity iodometrically

Amylase activity was assayed on the basis of the starch-iodometric method according to Zurcher & Hadorn (1972) with some modifications. By definition one unit of amylase activity (AA) equals mass of degradation (g) during one hour and express per 100g sample.

For determination a representative portion of sample (0.2 ± 0.001 g) was accurately weighted in a 100 mL glass test tube, dissolved in distilled water (electrical conductivity 10 μS cm^{-1}), held for 10 min with occasional shaking at room temperature and finally centrifuged. 20 mL from supernatant was placed in 50 mL test tube, in another test tube - 20 mL 0.5% starch solution. After holding in thermostat for 15 min at 40 °C both samples were mixed. After 0, 2, 6, 12, 20 and 30 minutes one mL of solution was put in 50 mL test tube with 30 mL of phosphate buffer solution with pH = 7.0 (58.9 g Na_2HPO_4 and 3.7 g citric acid dissolved in distilled water and filled till 1L) and 5 mL 0.05M iodine solution, filled with distilled water till 50 mL.

The total amylases activity (AA) was determined spectrophotometrically by absorption measurements at 565 nm and calculating according to the equation (1.):

$$AA = \frac{500 \cdot (A_0 - A_{30})}{A_0} \quad (1)$$

where: A_0 – absorption at t = 0 min; A_{30} – absorption at t = 30 min.

Determination of diastase activity with Phadebas

The unit of diastase activity, the Gothe unit, is defined as that amount of enzyme which will convert 0.01 gram of starch to the prescribed end-point in one hour at 40 °C under the conditions of test. Results are expressed in Gothe units per gram of malt.

The diastase activity of samples was measured with the Phadebas method. A tablet of an insoluble blue-dyed, cross-linked starch was used as the substrate for the degradation reaction. After dissolving 1.00 g of malt in the 0.1M acetate buffer (pH = 5.2) in a volumetric flask, 5.0 mL of the malt solution was transferred to the test tube and incubated in a water bath at 40 °C for a few minutes. A blank was prepared by adding 5.0 mL of the 0.1M acetate buffer (pH = 5.2) solution and was treated in the same manner as a sample solution. After placing the Phadebas tablets into both test tubes, a timer was started. The tubes were quickly removed from the water bath, stirred and then returned to the water bath. After 30 min, the reaction was terminated by adding 1.0 mL of 1 M sodium hydroxide solution. The mixture was stirred again and filtered. The absorbance of the sample was measured at 620 nm with deionised water as a reference. The absorbance of the blank was subtracted from that of the sample solution (DA_{620}).

The diastase activity, expressed as DN or diastase number, was calculated according to the equation (2):

$$DN = 28.2 \times \Delta A_{620} - 2.64^{\vee} \quad (2)$$

where: ΔA_{620} – sample absorbance – blank absorbance

Diastase activity was referred to as DN in the Schade scale, which corresponds to the Gothe scale number, or g, of starch hydrolysed per hour at 40 °C per 100 g of malt (Sak-Bosnar & Sakac, 2012).

Determination of α-amylase activity

α-amylase activity (AA) was measured using 'non-reducing-end blocked p-nitrophenyl maltoheptaoside' as substrate and following instructions from the Megazyme. One unit of amylase activity is defined as the amount of enzyme required to release one micromole of p-nitrophenol from p-nitrophenyl maltoheptaoside in one minute. This unit is termed a Ceralpha Unit (units g^{-1}). α-amylase activity was calculated according to the equation (3):

$$AA = \Delta A_{410} \cdot 382 \quad (3)$$

where: ΔA_{410} – sample absorbance – blank absorbance.

Viscosity measurements

For analyzing falling number and viscosity the rye malt was added to rye flour (malt concentration in sample 1.25%). To determine the Falling Number, 7 g of flour are heated with 25 ml of distilled water in a water batch for one minute to approximately 95 °C. The viscosity of the starch gel thus obtained is then determined by measuring the time the stirring rod takes to sink through the gel to the bottom of the measuring cylinder. The Falling Number is the sum of the stirring and sinking time. It is stated in seconds. The falling number and amylogram curve (80 g flour sample) were determined by methods 56–81B and 22–10, respectively. Peak viscosity was obtained from the amylogram curve in Brabender units (BU). The amylograph value or peak viscosity is inversely correlated with amylase activity.

Statistical analysis

The results (mean values, standard deviation, *P*-value) were processed by mathematical and statistical methods. Significance was defined at $P < 0.05$. Statistical analyses were done using standard Microsoft Excel software.

RESULTS AND DISCUSSION

Determination of total amylases activity iodometrically

The total activity of amylases was determined iodometrically. Obtained results showed that microelement selenium has positive influence on the amylases activity (Fig. 1). The relative increase of enzyme activity was for 13.4% compared with the control sample. Analysing the role of another microelement – copper, we can conclude that presence of copper decreases enzyme activity (Fig. 1, samples III–V). The highest

decrease of amylases activity was observed analysing samples with copper concentration 5 and 10 mg L^{-1} (sample IV and V) – for 16% and 23% respectively. It can be explain with different antagonistic or synergistic relationships between microelements (Siedlecka, 1995; Ouzounidou et al., 1997).

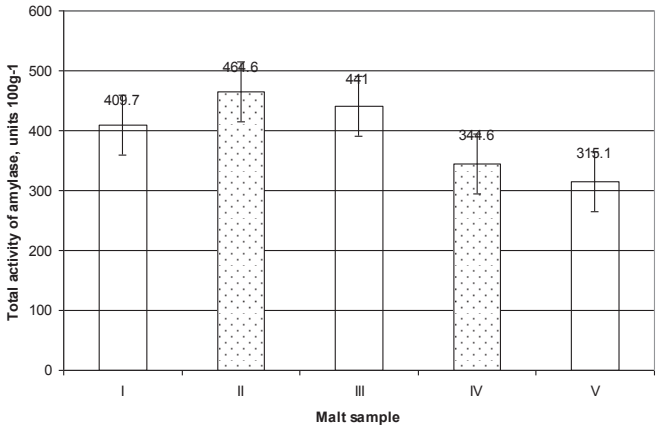

Figure 1. Total activity of amylases in rye malt samples. I – control; II – Se (8.5 mg L^{-1}); III – Se (8.5 mg L^{-1}) and Cu (3 mg L^{-1}); IV – Se (8.5 mg L^{-1}) and Cu (5 mg L^{-1}); V – Se (8.5 mg L^{-1}) and Cu (10 mg L^{-1}).

It was interesting to compare the activity of barley and rye malt. The obtained results (Fig. 2) showed that amylases in barley malt 'start to work' after 6 minutes from the beginning of reaction but enzymes in rye malt the maximum of activity reached only after 30 minutes.

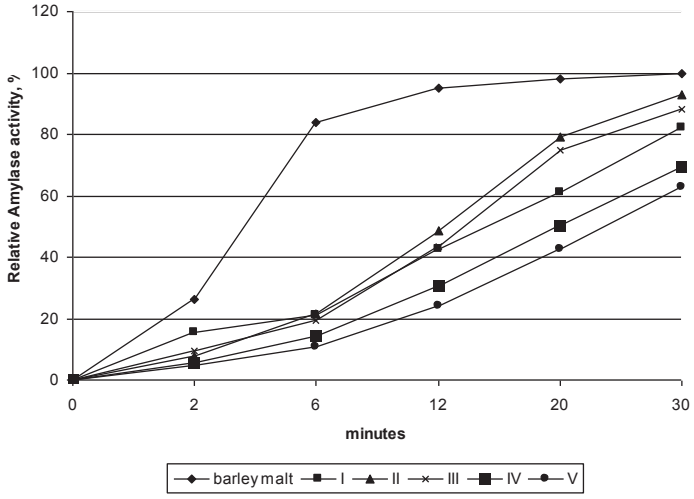

Figure 2. Relative amylase activity (%) in barley and rye malt. I – control; II – Se (8.5 mg L^{-1}); III – Se (8.5 mg L^{-1}) and Cu (3 mg L^{-1}); IV – Se (8.5 mg L^{-1}) and Cu (5 mg L^{-1}); V – Se (8.5 mg L^{-1}) and Cu (10 mg L^{-1}).

The results of research (Fig. 2) show evidence that the presence of selenium (sample II) promotes the activity of enzyme and this effect start to appear after 6 minutes from the beginning of reaction.

Determination of α-amylase activity with Megazyme test kid

There are several enzymes reacting as starch hydrolases, but the most important is the α-amylase. Determination of α-amylase activity with Megazyme test kid was used to be sure that the microelement copper has influence on the α-amylase activity. For determination of α-amylase, the well-known Ceralpha method was used. The results of research (Fig. 3) show that the highest activity of α-amylase was determined for second sample – rye malt with selenium additives. In this case the enzyme activity was two times higher comparing with the control sample. Therefore we can conclude that microelement selenium promoted enzyme activity and the changes were significant ($P < 0.05$). Numerous studies have demonstrated the Se beneficial effect including growth promoting activities (Terry et al., 2000), have shown enhanced resistance to certain abiotic stresses, drought (Kuznetsov et al., 2003), salinity, chilling and UV-radiation (Valkama et al., 2003).

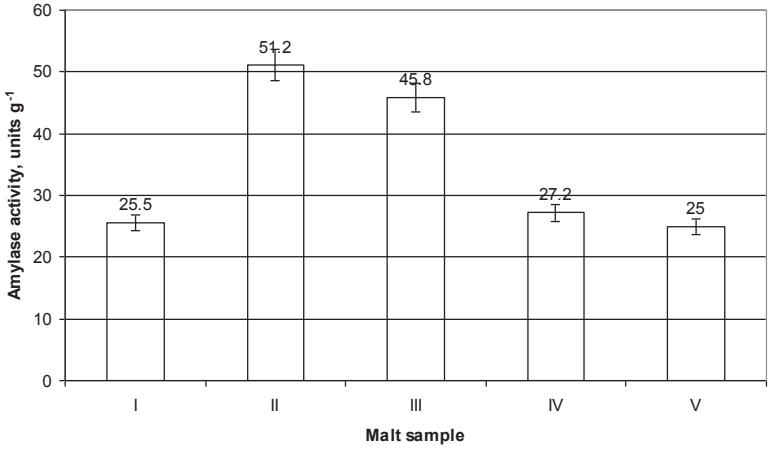

Figure 3. Determination of α-amylase activity with Megazyme test kid. I – control; II – Se (8.5 mg L^{-1}); III – Se (8.5 mg L^{-1}) and Cu (3 mg L^{-1}); IV – Se (8.5 mg L^{-1}) and Cu (5 mg L^{-1}); V – Se (8.5 mg L^{-1}) and Cu (10 mg L^{-1}).

Analysing the activity of α-amylase in rye malt with two different microelement additives, a conclusion can be drawn that a negative influence of microelement copper was observed. All analysed copper concentrations, except Cu 3 mg L^{-1}, significantly reduce the effect of selenium. If the concentration of copper is higher than 3 mg L^{-1}, the promotion effect of selenium was not observed. It can be explained with the copper toxic influence on the enzyme biosynthesis. From previous studies (Antoņenko et al, 2013a; Mihoub et al., 2005) it is known, that copper may inhibit biological processes in grain during germination. Further Cu in excess is strongly phytotoxic and may alter membrane permeability, chromatin structure, protein synthesis, enzyme activities, photosynthetic

and respiratory processes, and may activate senescence (Vinit-Dunand et al., 2002). The obtained results show similar tendency as previous results (Fig. 1).

Determination of enzyme activity with Phadebas test

There are several determination methods for enzyme activity. One of them is Phadebas Amylase test. Despite the fact that this method generally can be applied to honey samples, it may also be used for determination of diastase activity in another sample.

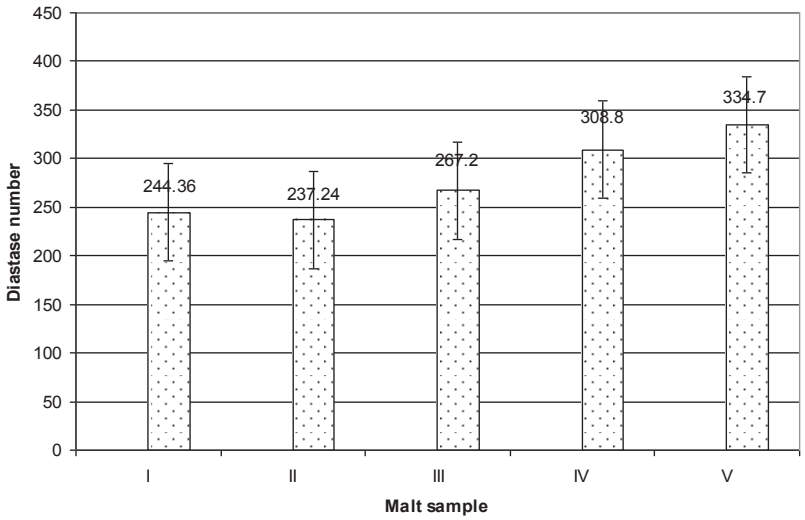

Figure 4. Determination of α-amylase activity with Phadebas test. I – control; II – Se (8.5 mg L^{-1}); III – Se (8.5 mg L^{-1}) and Cu (3 mg L^{-1}); IV – Se (8.5 mg L^{-1}) and Cu (5 mg L^{-1}); V – Se (8.5 mg L^{-1}) and Cu (10 mg L^{-1}).

The obtained results (Fig. 4) showed that presence of microelement selenium slightly decreases the enzyme activity (about 3%) comparing with the control sample, but selenium together with copper promoted the enzyme activity. The presence of Cu significantly ($P < 0.05$) increases the activity of α-amylase and the highest increase was observed at following microelements concentration: Se 8.5 mg L^{-1} and Cu 10 mg L^{-1}. We can see that these experimental results are inversely proportional to results obtained with previously used methods. We think, that it can be explained by method's specificities. The final product of reaction is blue water soluble substance, determined photometrically at 620 nm. As it is known, copper compounds are also mainly in blue color and therefore it will influence the final results.

The falling number and viscosity measurements

Rye grain contains enzymes that attack all of its major constituents, and especially the starch-degrading amylases play a key role in relation to the baking quality of the flour (Seibel, Weipert 2001). To test the effect of different rye malt samples enriched with two microelements selenium and copper on the technological properties of rye flour, the flour samples with 1.25% additives of rye malt were prepared. The effects of

the rye malt, enriched with microelements were apparent in falling numbers and the results of amylogram (Table 1). The rye flour was used as control.

Table 1. The influence of microelements on the rye malt flour technological properties

Sample	Falling number, s	Amylograph peak viscosity, BU
Rye flour	200	616
Rye flour + rye malt without additives	171	553
Rye flour + rye malt enriched with Se 8,5 mg L^{-1}	152	472
Rye flour + rye malt enriched with Se 8,5 mg L^{-1} and Cu 3 mg L^{-1}	160	570
Rye flour + rye malt enriched with Se 8,5 mg L^{-1} and Cu 5 mg L^{-1}	158	584
Rye flour + rye malt enriched with Se 8,5 mg L^{-1} and Cu 10 mg L^{-1}	176	599

Falling number (FN) and amylogram characterize the properties of starch and α-amylase activity. The greatest decrease in viscosity and falling number often corresponds to the high activities of hydrolytic enzymes in rye grain (Salmenkallio-Marttila & Hovinen, 2005). It is known that the rye flour normally has a FN in the range of 150 s, and in dry years the FN are 300 s and higher (Seibel & Weipert 2001; Hansen et al. 2004), the minimum FN requirement for intervention of rye in the EU was 100 s. Hansen, 2004 reported, if the FN is too low results in pasty and unacceptable bread.

The obtained experimental results confirm these conclusions. Different rye malt additives decrease falling number from 12% till 24%. The lowest falling number was determined for flour sample containing rye malt enriched with selenium. Taking into account previous experimental results it is evident that rye malt with selenium additives has the highest enzyme activity. These results also show that microelement copper has negative influence on the baking quality of rye flour - falling number and peak viscosity increase comparing with the control sample. The higher the concentration of copper, the greater increase of viscosity was observed.

CONCLUSIONS

The additives of microelement selenium significantly increase the total activity of amylases (iodometrically determination) and activity of α-amylase (determined with Megazyme test) in rye malt. Using two microelements Cu and Se for rye malt production, the activity of amylases decreases, moreover, the higher the concentration of copper, the less activity was observed. Phadebas Amylase test gave inverse results, because the presence of Cu significantly increases results. Analysing technological properties of rye flour with rye malt additives, the lowest falling number and viscosity were determined for samples enriched with selenium. The malt samples enriched with copper increase falling number and viscosity that means lower amylase activity.

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