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Nucleic Acid Degrading Enzymes of Barley Malt

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Abstract

Ten cultivars of malting barley grown at four locations were malted and assayed for six enzymes involved in the degradation of nucleic acids. Among these enzymes were deoxyrinonuclease, ribonuclease, phosphodiesterase, 3′- and 5′- nucleotidases and phosphomonoesterase. Activities of all enzymes in five-day malts were significantly affected by variety and location of growth. The average levels of ribonuclease, deoxyribonuclease, 3′-nucleotidase and 5′-nucleotidase of 80 five-day malts were 11.2, 5.7, 5.6 and 1.2 units per gram of malt, respectively. Six-rowed barley malts contained higher levels of deoxyribonuclease, phosphodiesterase and 3′-nucleotidase than those of two-rowed barley malts, while two-rowed barley malts contained significantly higher ribonuclease levels than those of six-rowed barley malts.

Key words: malt, nuclease, phosphatase

Introduction

The rate of yeast growth during fermentation is dependent upon the ability of yeast to synthesize the essential enzymes involved in metabolism. The synthesis of these enzymes by yeast is dependent on the low molecular weight nitrogenous compounds of wort which are degradation products of both protein and nucleic acids[13].

Some slow fermentations by ale yeast could be directly attributed to deficiencies in inositol and in nucleic acid bases[25]. Slowly fermenting worts contained less than 10mg/*l* of adenine compared with approximately 40mg/*l* in normally fermenting wort, and the rate of fermentation could be stimulated by the addition of nucleobases. It has recently been suggested that the addition of nucleobases(adenine, cytosine, guanine, thymine and uracil) to synthetic media inoculated with *Saccharomyces carlsbergensis* had a significant effect on yeast growth[15].

It is also known that nucleic acids have been implicated in turbidity formation[11] and the presence of soluble, high molecular weight nucleic acid in the wort may lead to poor physical stability in the resulting beer[2]. The nucleotides such as guanosine monophosphate and inosine monophosphate have been recognized as flavor enhancing substances[5].

The levels of nucleotides, nucleosides and free purine and pyrimidine bases have been reported for malt extracts, wort and beers and the effects of processing conditions on these levels have been examined[6,27].

Enzymes which catalyze the breakdown of nucleic acids by hydrolysis of phosphodiester bonds have been widely founds in plants[5]. Some, the ribonucleases(RNases), are quite specific for RNA, while the others, the deoxyribonucleases(DNases), act only on DNA. Phosphodiesterase(PDase) in an exonuclease which acts on a polynucleotide chain from the 3′-end, releasing 5′-nucleotides. The phosphomonoesterase(PMase) act on polynucleotides or

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oligonucleotides with a terminal phosphate group or on a mononucleotide to liberate inorganic phosphate. 3′-nucleotidase and 5′-nucleotidase attack the 3′- and 5′ phosphate groups of a nucleotide, respectively, yielding the phosphate group and the corresponding nucleoside.

A number of reports are present in the literature regarding the presence of these enzymes in germinating barley. The presence of DNase in germinating barley was reported by Brawerman and Chargaff[4] and this enzyme was partially purified by Liao[17] and Sasakuma and Oleson[23]. Liao[17] isolated a DNase from germinating barley seeds which had enzymatic activity very similar to the DNase found in animals. He separated the enzyme into four distinct forms. Sasakuma and Oleson[23] also partially purified nuclease I from malted barley. This enzyme was a sugar-unspecific endonuclease which degraded native DNA, denatured DNA and RNA and it also possessed 3′-nucleotidase activity.

RNase was partially purified from dried malt rootlets by Fiers and Vandendriessche[8]. Pietrzak et al[20] also isolated three enzymes possessing RNase activity from barley seeds. Two of the enzymes studied were specific for RNA and the third was able to hydrolyze both RNA and DNA. They confirmed that the enzyme hydrolyzing RNA and DNA was nuclease I and that the two other enzymes specific for RNA were RNase I and RNase II. Srivastava[24] investigated the effect of gibberellic acid on the ribonuclease activity of germinating barley seeds. As germination progressed, there was a consistant increase in the acitivity of ribonuclease in both control and GA -treated seeds, the latter showing higher activity throughout a 96hr germination.

The presence of a phosphodiesterase in germinating barley has been reported by Brawerman and Chargaff[4] and Fiers and Vandendriessche[8] and it was partially purified by Georgatsos[9] and Holbrook[12].

The presence of PMase in barley malt was reported by Brawerman and Chargaff[4]. Nakagiri[18] reported that barley rootlets contained 3′-nucleotidase acitivity and weak 5′-nucleotidase activity.

Recently, it was reported that mature barley contained low levels of nucleases and phosphatases and the activities of all of the enzymes increased during germination[14,21]. All of the enzymes were quite stable during kilning at 45°. Phosphatases and PDase showed good temperature stability during kilning at 65 and 85℃, while DNase and RNases showed slight losses at these temperatures[14]. Nucleases and phosphatases were affected to only a slight degree during low temperature(45℃) of mashing but activity was lost rapidly as the mash temperature rose to 70℃[14].

It has recently been suggested that the level of nucleases and nucleotidases in malting barley is a varietal characteristic[21]. The main purpose of this study was to assess the variation in nucleases and phosphatase activities occurring in malting barley varieties and types and to determine to what extent the environment influences this variation.

Materials and Methods

Samples

Ten barley cultivars grown at four locations(Fargo, Langdon, Minot and Williston, ND, U.S.A.) in 1982 and 1983 were supplied by the North Dakota Agricultural Experiment Station. These included six cultivars of six-rowed barley(Morex, Larker, Glenn, Azure, Park and ND 5569) and four cultivars of two-rowed barley(Clark, Harrington, ND 5698 and ND 4994).

Preparation of Malted Barley

A modification of the malting procedure described by Banasik and co-workers[1] was used in this study. Individual samples(60g, dry basis) were steeped at 16℃ to 45% moisture, then were germinated at 16℃ and 100% R.H. for five days. The entangled rootlets of the germinated samples were

separated daily during germination. Upon completion of germination, the samples were kilned at 45℃ for 24hr, conditions under which almost all enzyme activity could be recovered[14]. After kilning, rootlets were removed from the dry malt and it was finely ground in the coffee mill(Model 203, Robert Krups, North America, Allendale, NJ, U.S.A.) for 45 sec.

Preparation of Enzyme Extract

Five grams of ground malt was extracted with 50*ml* of Tris-acetate buffer for 1hr at 4℃. RNase was extracted with 0.05M buffer(pH 8.0), DNase and PDase were extracted with 0.1M buffer(pH 7.0) and 3′- and 5′ -nucleotidase and PMare were extracted with 0.1M buffer(pH 5.0). The optimum pH and ionic strength for extraction of each enzyme were determined in a preliminary study.

Preparation of Partially Degraded DNA

Partially degraded DNA was prepared from salmon sperm DNA(Sigma Chemical Co., St. Louis, MO, U.S.A.) by the method of Oleson and Koerner[19]. The DNA was incubated with pancreatic DNase until approximately 19% of the material was rendered acid soluble and the acid -insoluble material was precipitated by the addition of trichloroacetic acid and isolated by centrifugation. The final preparation was dried overnight at room temperature.

Preparation of RNA Substrate

Torula yeast RNA(Sigma, Type VI) was dissolved(10mg/m*l*) in 0.1M Tris-acetate buffer(pH 7.4), subjected to two phenol extraction to remove contaminating protein, and precipitated with two volumes of ethanol[3]. This material was dissolved in a small volume of Tris buffer(2% phenol+0.01M Tris-hydrochloride, pH 7.9) and applied to a column of Sephadex G-75. The column was eluted with Tris buffer and fractions containing RNA fragments of the highest molecular weight were pooled. The purified RNA was precipitated with two volumes of ethanol, and isolated by centrifugation and dried at room temperature.

DNase Assay

The assay used for DNase activity was a modification of the procedure of Lehman[16]. The reaction mixture contained 1mg of partially degraded DNA in 1m*l* 0.05M Tris-acetate buffer(pH 5.8) and 100μl of a 1:4 dilution(with substrate buffer) of the extract. After incubation at 37℃ for 30 min, the reaction was terminated by chilling to 0℃ and adding 1m*l* of 1N $HClO_4$. The mixture was allowed to stand at 0℃ for 10 minutes before being centrifuged at approximately 5,000 x g. The absorbance of the supernatant solution was determined at 260*nm*. One unit of DNase activity is defined as the amount of enzyme that catalyzed the release of 1.0 μ mole of acid soluble product per min under the conditions of the assay. Activity is expressed as units per gram of malt.

RNase Assay

RNase activity was estimated in the manner described for DNase. The reaction mixture consisted of 0.5m*l* of 0.5% RNA in water(dialyzed against water overnight), 1.0m*l* of 0.05M sodium acetate buffer(pH 5.5) and 50μl of enzyme extract.

Phosphodiesterase Assay

The reaction mixture contained 1.0m*l* of 1mM *p* -nitrophenyl-5′-thymidylate in 0.05M Tris-acetate buffer(pH 6.0) and 50m*l* of a 1:10 dilution(with substrate buffer) of extract. After incubation at 37℃ for 30 min, the reaction was terminated by the addition of 0.5m*l* of 0.3N sodium hydroxide. The absorbance at 420*nm* due to enzymatically produced *p*-nitrophenol was determined. Activity is expressed as μ mole of *p*-nitrophenol produced per min per gram of malt.

3′-and 5′ -nucleotidases and Phosphomoesterase Assay

The substrate solutions for 3′- and 5′-nulce-

otidases and phosphomonoesterase consisted of 2.5mM 3′-AMP, 2.5mM 5′-AMP or 1.25 mM β-glycerophosphate, respectively, each in 0.05M Tris-acetate buffer(pH 6.0). The reaction mixtures consisted of 1.0m*l* of substrate and 100m*l* of a 1:10 dilution(with substrate buffer) of the extract for the 3′-nucleotidase assay or 200μ*l* of a 1:10 dilution of the extract for the 5′-nucleotidase assay. The reaction mixture for phosphomonesterase assay consisted of 2.0m*l* of substrate and 500μ*l* of a 1:10 dilution of the extract. For each assay, the reaction mixture was incubated at 37℃ for 30 min. The reactions were terminated by chilling to 0℃ and the digestion mixtures were immediately analyzed for inorganic phosphate as described by Chen[7]. One unit of activity is defined as the amount of enzyme required to liberate on μ mole of phosphate per min per gram of malt.

Results and Discussion

Nuclease Activities of Malt

Ten malting barley varieties were obtained from four locations over two years and constituted 80 treatment combinations. The statistical analysis used for nucleic acids degrading enzymes in malt can be considered to be a mixed model. Barley variety was treated as a fixed factor, while the factors year and location were treated as random. Thus, while any conclusions drawn regrading varietal effects will apply only to the ten varieties tested, conclusions regarding the lacation and annual effects can be made in general terms, i.e., they apply to any year and location, and not to specific years or locations. Data were analyzed by computer using the statistical analysis system(SAS) as described by the SAS Institute[22].

Table 1 shows the results of an analysis of variance of data obtained from ten varieties grown at four locations over two crop years. Both variety and location of growth were found to have a statistically significant effect on the level of barley malt deoxyribonuclease(DNase) and ribonuclease(RNase), but there was no significant annual effect. Prentice[21] examined the influence of variety on nucleases and phosphatases and nucleosidases of fourteen barley varieties grown at one location in Idaho. He concluded that there appeared to be a varietal influence on nucleic acid degrading enzymes. However, because of limited sample size and lack of statistical analysis, these results must be viewed as being merely suggestive.

Table 1. Analysis of variance; Effect of variety, location and year on nuclease activity (units per gram of malt)

Source	df	DNase		RNase	
		Sum of squares	F	Sum of squares	F
Variety	9	121.6110	25.64*	446.2843	13.99*
Location	3	21.9003	13.85*	94.4528	8.88*
Varietyx Location	27	31.2735	2.20*	128.4738	1.34
Year	1	0.0003	0.0	3.7347	1.05
VarietyxYear	9	3.4485	0.73	28.3980	0.89
Locationx Year	3	40.4203	25.57*	175.0658	16.46*
VarietyxLocationxYear	27	7.4860	0.53	83.8046	0.88
Error	80	41.1600		283.6049	

* Significant at the 1% level.

The variety x location interaction and location x year interaction had a highly significant effect on DNase activity, while other interactions were not significant. Plots were prepared to aid in the evaluation of each of the significant interactions[10]. From an evaluation of these plots, it can be concluded that, while sighnificant differences in malt DNase activities exist between the varieties examined, the magnitude of these differences does not remain constant over locations. A variety that exhibits high activity at one location will not necessarily be expected to have a relatively high activity at other locations. However, a variety that exhibits high activity in one year will be expected to have a relatively high activity in other years, because the interaction between variety and year did not show a significant effect on DNase activity.

Table 2 shows the results of Duncan's multiple

range test for malt nuclease activities. Morex and Park(six-rowed barleys) were, on the average, significantly higher in DNase activity than the other varieties studied and Harrington and ND 4994(two-rowed barleys) were significantly lower than the rest. DNase activity of five-day malts, on the average, ranged from 4.63 to 7.49 units per gram of malt.

Neither interaction between variety and location nor interaction between variety and year had a significant effect on RNase activity. Thus, significant differences in malt RNase activity exist between the varieties examined and these differences will remain constant over locations and years. Harrington(two-rowed barley) showed the highest level of malt RNase activity in all cases, and Larker(six-rowed barley) showed the lowest level of RNase activity in all cases(Table 2). The average RNase contents of five-day malts ranged from 8.62 to 14.98 units per gram of malt. Five-day malts contained twice as much RNase activity as DNase activity.

Variety, location and year had significant effect on phosphodiesterase(PDase)(Table 3). Since none of the interactions was found to be significant, the three factors, variety, year and location, act independently of one another. The simple effects of a given factor will thus be the same for all levels of other factors studied.

Table 2. Duncan's multiple range tests for nuclelase activity[a)]

	N	Mean[b)] DNase	RNase	PDase
Variety				
Park	16	7.49^{a}	10.78^{cd}	0.91^{a}
Morex	16	7.19^{a}	11.29^{c}	0.89^{a}
Glenn	16	5.78^{b}	10.72^{cd}	0.71^{bc}
Larker	16	5.76^{b}	8.62^{e}	0.88^{a}
Clark	16	5.71^{b}	12.73^{b}	0.77^{b}
ND 5569	16	5.54^{bc}	12.07^{bc}	0.69^{c}
Azure	16	5.51^{bc}	10.95^{c}	0.70^{c}
ND 5698	16	5.04^{cd}	10.70^{cd}	0.72^{bc}
Harrington	16	4.95^{d}	14.98^{a}	0.67^{c}
ND 4994	16	4.63^{d}	9.43^{de}	0.67^{c}
Type				
Six-row	96	6.21^{a}	10.74^{b}	0.80^{a}
Two-row	64	5.08^{b}	11.96^{a}	0.71^{b}

a) In units per gram of malt
b) Means within the same group are not significantly different at P=0.95

Table 3. Analysis of variance; Effect of variety, location and year on pdase and pmase activity (units per gram of malt)

Source	df	PDase Sum of squares	F	PMase Sum of squares	F
Variety	9	1.3230	18.05**	1.7059	2.42*
Location	3	0.3018	12.35**	0.2249	0.96
VarietyxLocation	27	0.2744	1.25	0.7440	0.35
Year	1	0.0501	6.15**	0.0012	0.01
VarietyxYear	9	0.0734	1.00	0.3244	0.46
LocationxYear	3	0.0217	0.89	0.3250	1.38
VarietyxLocation xYear	27	0.0910	0.41	0.7147	0.34
Error	80	0.6515		6.2686	

* Significant at the 5% level.
** Significant at the 1% level.

Five-day malts contained the relatively low levels of phosphodiesterase activity. The average PDase activity ranged from 0.67 to 0.91 units per gram of malt(Table 2). Park, Morex and Larker(six-rowed barleys) showed high levels of malt PDase in all cases and Harrington and ND 4994(two-rowed barleys) showed low levels of activity.

Six-rowed barleys contained higher levels of DNase and PDase than did two-rowed barleys when barley type(two-rowed and six-rowed) was tested as a factor(Table 2). However, two-rowed barleys contained significantly higher levels of RNase than did six-rowed barleys.

Phosphatase Activities of Malt

Variety, location and year were found to have highly significant effect on malt 3′- and 5′-nucleotidase(Table 4). Only the location x year interaction showed a significant effect on 3′-nucleotidase activity. Neither interaction between

Table 4. Analysis of variance; Effect of variety, location and year on nucleotidase activity (units per gram of malt)

Source	df	3-Nucleotidase Sum of squares	F	5-Nucleotidase Sum of squares	F
Variety	9	28.6519	3.98**	1.2039	2.30**
Location	3	8.7122	3.63*	0.9908	5.67**
VarietyxLocation	27	14.4891	0.67	0.6759	0.43
Year	1	7.7374	9.68**	1.4655	25.17**
VarietyxYear	9	2.2488	0.31	0.3659	0.70
LocationxYear	3	9.9109	4.13**	0.3698	2.12
VarietyxLocation xYear	27	29.5762	1.37	1.1142	0.71
Error	80	63.9388		4.6579	

* Significant at the 5% level.
** Significant at the 1% level.

Table 5. Duncan's multiple range tests for phosphatase activity[a)]

	N	Mean[b)] 3'-Nucleotidase	5'-Nucleotidase	PMase
Variety				
Morex	16	6.13^{a}	1.35^{a}	1.16^{a}
Park	16	5.98^{ab}	1.23^{ab}	0.95^{abc}
Azure	16	5.94^{ab}	1.24^{ab}	1.03^{abc}
Larker	16	5.87^{ab}	1.18^{ab}	1.01^{bc}
Glenn	16	5.77^{ab}	1.20^{ab}	0.89^{bc}
Clark	16	5.74^{ab}	1.24^{ab}	1.02^{abc}
ND 5569	16	5.48^{abc}	1.06^{b}	0.82^{c}
Harrington	16	5.37^{bc}	1.30^{a}	0.89^{bc}
ND 4994	16	4.93^{c}	1.19^{ab}	1.10^{ab}
ND 5698	16	4.82^{c}	1.07^{b}	0.86^{c}
Type				
Six-row	96	5.86^{a}	1.21^{a}	0.98^{a}
Two-row	64	5.21^{b}	1.20^{a}	0.97^{a}

a) In units per gram of malt
b) Means within the same group are not significantly different at P=0.95

variety and location nor interaction between variety and year appeared to have a significant effect on the activities of these enzymes. Thus, the differences between varieties remain constant over growth locations and years.

3'-Nucleotidase activity of five-day malt ranged from 4.82 to 6.13 units per gram of malt and 5'-nucleotidase activity ranged from 1.06 to 1.35 units per gram of malt(Table 5). On the average, the five-day malt contained 4.6 times as much 3'-nucleotidase as 5'-nucleotidase activity. Morex(six-rowed barley) showed high levels of both 3'- and 5'-nucleotidase activity and ND 4994 and ND 5698(two-rowed barleys) showed the relatively low levels.

Phosphomonoesterase(PMase) activity of five-day malts were significantly influenced by variety, but not by location or year of growth(Table 3). Thus, significant differences in PMase activity can be expected between varieties and again, because none of the interactions were significant, the differences between varieties will remain constant over growth locations and years. The average PMase contents of five-dya malts ranged from 0.82 to 1.16 units per gram of malt. Morex(six-rowed barley) was distinctly higher in malt PMase than the other samples and ND 5569(six-rowed barley) and ND 5698(two-rowed barley) were significantly lower than the rest.

Six-rowed barleys exhibited significantly higher levels of malt 3'-nucleotidase activity than did two-rowed barleys, but barley type did not have a significant effect on 5'-nucleotidase and PMase activities.

Morex(six-rowed barley), which is currently an acceptable malting variety in the U.S., was on average, over locations and years, significantly higher in most nuclease and phosphatase activities than the other varieties studied, while ND 5698 and ND 4994(two-rowed barleys) were significantly lower in most of the enzymes studied.

요 약

네 지역에서 2년 동안 재배된 10종의 맥주맥을 제맥하여 맥아중의 핵산분해에 관련된 6 종류의 효소를 측정하였다. 측정된 효소는 데옥시리보핵산 가수분해효소, 리보핵산 가수분해효소, 포드포디에스테르 가수분해효소, 3'-뉴클레오티드 가수분해효소, 5'-뉴클레오리드 가수분해효소와 포스포모노 에스테르 가수분해효소 이었다. 5

일 동안 발아시킨 맥아속의 효소는 맥주맥의 품종이나 재배지역에 따라 크게 영향을 받았고, 현재 장려품종으로 재배되고 있는 몇몇 품종은 이러한 효소들을 상당량 함유하고 있었다. 80 시료의 리보핵산 가수분해효소, 데옥시리보핵산 가수분해효소, 3′-뉴클레오리드 가수분해효소와 5′-뉴클레오리드 가수분해효소의 평균 함량은 각각 11.2, 5.7, 5.6과 1.2 units 이었다. 6조 맥의 맥아는 2조 맥의 맥아보다 데옥시리보핵산 가수분해효소, 포스포디에스테르 가수분해효소와 3′-뉴클레오리드 가수분해효소를 더 많이 함유하고 있었고, 2조 맥의 맥아는 리보핵산 가수분해효소 함량에서 6조 맥의 맥아보다 더 높았다.

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