PREPARATION OF 5′-GMP-RICH YEAST EXTRACTS FROM SPENT BREWER'S YEAST

PHANIDA SOMBUTYANUCHIT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2000

ISBN 974-664-992-2

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was submitted to the Faculty of Graduate studies, Mahidol University
for the degree of Master of Science (Biotechnology)
on
October 27, 2000.

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ACKNOWLEDGMENT

I would like to express my deepest sincere and appreciation to my advisor, Assoc. Prof. Dr. Manop Suphantharika for his supervision, continuous guidance and encouragement throughout the course of this study which enable me to attain this thesis successfully. All his kindness, generosity and sympathy will be long remembered with respect.

I do greatly appreciate the members of advisory committee, Assoc. Prof. Dr. Saiyavit Varavinit and Dr. Pairoj Luangpituksa for their valuable suggestions, expert comments and criticisms with kindness about the work of my thesis.

I am also deeply indebted to Assoc. Prof. Dr. Sujin Shobsngob and Dr. Cornel Verduyn for their valuable advice and comments as well as their kindness and understanding throughout the study.

Special thanks are given at all members of the room BT 207 and BT 210 for their helps, friendship and encouragement and also to my all fellows in the Department of Biotechnology for their helpful corporations with sincerity and friendliness and others who have not been mentioned here for their encouragement.

Finally, I would like to special express my gratitude and deepest appreciation to my parents and members in my family, for their love, sincere, intention, encouragement, understanding support throughout my life which will never be forgotten.

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3936487 SCBT/M : MAJOR : BIOTECHNOLOGY ; M. Sc. (BIOTECHNOLOGY)

KEY WORDS : 5′-GMP / BREWER'S YEAST / YEAST EXTRACT / MALT ROOTLETS

PHANIDA SOMBUTYANUCHIT : PREPARATION OF 5′-GMP-RICH YEAST EXTRACTS FROM SPENT BREWER'S YEAST. THESIS ADVISORS : MANOP SUPHANTHARIKA, Ph.D., SAIYAVIT VARAVINIT, Dr.Ing., PAIROJ LUANGPITUKSA, D.Arg.Sc, CHOCKCHAI THEERAKULKAIT, Ph.D., 117 P. ISBN 974-664-992-2

Spent brewer's yeast was autolysed and used as a raw material for the preparation of 5′-GMP-rich yeast extracts. Fresh malt rootlet extract, dried malt rootlets extract and rice rootlets extract were analyzed for specific activity of 5′-phosphodiesterase for use as a source of enzyme. The highest specific activity was obtained from fresh malt rootlet extract followed by dried malt rootlets and rice rootlets, respectively. Dried malt rootlets were used as a source of 5′-phosphodiesterase due to their lowest cost. Before the dried malt rootlet extract was used, it was concentrated by ammonium sulfate precipitation at 40-80 % saturation and then was pre-treated to inactivate 5′-nucleotidase. The optimum pretreatment conditions were heating at 65°C for 30 min or 70°C for 7 min. The effects of autolysis time (at 15% w/v solids, pH 5, 50°C), phosphodiesterase concentration and incubation period on 5′-GMP content were examined. The suitable autolysis time was 8-12 hrs. The preferable enzyme treatment period was in the range of 8-14 hrs. Longer autolysis and enzyme incubation periods caused a decrease in the 5′-GMP content from 0.7-0.9%(w/w) to 0.2-0.4 %(w/w). The 5′-GMP content in extracts from debittered yeast using 2 % sodium carbonate was not different form non-debittered yeast. The highest 5′-GMP content in yeast extract was 0.93 %(w/w), obtained with a phosphodiesterase concentration of 160 units/100 ml of yeast extract (5% w/v).

3936487 SCBT/M : สาขาวิชา : เทคโนโลยีชีวภาพ ; วท.ม (เทคโนโลยีชีวภาพ)

พนิดา สมบัติยานุชิต : การเตรียมสารสกัดที่มีปริมาณ 5'-GMP สูงจากยีสต์ที่ใช้แล้วจากอุตสาหกรรมการผลิตเบียร์ (PREPARATION OF 5'-GMP-RICH YEAST EXTRACTS FROM SPENT BREWER'S YEAST). คณะกรรมการควบคุมวิทยานิพนธ์ : มานพ สุพรรณธริกา, Ph.D., ไสยวิชญ์ วรวินิต, Dr. Ing., ไพโรจน์ หลวงพิทักษ์, D.Arg.Sc, โชคชัย ธีรกุลเกียรติ, Ph.D. 117 หน้า. ISBN 974-664-992-2

ยีสต์ที่ใช้แล้วจากอุตสาหกรรมการผลิตเบียร์ถูกนำมาศึกษาถึงสภาวะที่เหมาะสมในการเตรียมสารสกัดที่มีปริมาณ 5'-GMP สูง รากข้าวมอลท์แห้ง สด และรากข้าวเจ้าถูกนำมาศึกษาถึงปริมาณ specific activity ของ 5'-phosphodiesterase เพื่อเลือกใช้เป็นแหล่งของเอนไซม์ สารสกัดจากรากข้าวมอลท์สดให้ปริมาณเอนไซม์มากที่สุด รองลงมาคือรากข้าวมอลท์แห้งและรากข้าวเจ้าตามลำดับ รากข้าวมอลท์แห้งถูกเลือกนำไปใช้เป็นแหล่งของ 5'-phosphodiesterase เนื่องจากมีราคาต่ำ ก่อนที่สารสกัดจากรากข้าวมอลท์แห้งจะถูกนำไปใช้จะถูกนำไปเพิ่มความเข้มข้นด้วยการตกตะกอนด้วยแอมโมเนียมซัลเฟทที่ความเข้มข้นอิ่มตัว 40-80 % จากนั้นจึงถูกนำไปยับยั้งการทำงานของเอนไซม์ 5'-nucleotidase โดยสภาวะที่เหมาะสมในการยับยั้งคือการให้ความร้อนที่อุณหภูมิ 65 องศาเซลเซียส เป็นเวลา 30 นาที หรือ 70 องศาเซลเซียส เป็นเวลา 7 นาที ในการเตรียมสารสกัดที่มีปริมาณ 5'-GMP สูงครั้งนี้ ได้ศึกษาถึงความเหมาะสมของระยะเวลาในการย่อยสลายตัวเองของยีสต์ (ที่ปริมาณของแข็งของยีสต์ 15 % w/v, ความเป็นกรดด่าง 5, อุณหภูมิ 50 องศาเซลเซียส), ความเข้มข้นของเอนไซม์ 5'- phosphodiesterase และระยะเวลาในการทำปฏิกริยาของเอนไซม์ โดยพบว่าระยะเวลาที่เหมาะสมต่อการย่อยสลายตัวเองของยีสต์คือ 8-12 ชั่วโมง ระยะเวลาที่เหมาะสมของการย่อยสลายด้วยเอนไซม์คือ 8-14 ชั่วโมง นอกจากนี้ยังพบว่าการย่อยสลายตัวเองของยีสต์และการทำปฏิกริยาของเอนไซม์ที่นานขึ้น มีผลทำให้ปริมาณ 5'-GMP ลดลงจาก 0.7-0.9 %(w/w) เป็น 0.2-0.4 %(w/w) ปริมาณ 5'-GMP ในสารสกัดจากยีสต์ที่ผ่านกระบวนการกำจัดความขมด้วย 2% โซเดียมคาร์บอเนตให้ผลไม่แตกต่างจากการไม่กำจัดความขม ปริมาณสูงสุดของ 5'-GMP ในสารสดกัดจากยีสต์คือ 0.93 % w/w ได้จากปฏิกริยาที่ใช้เอนไซม์ 160 unit ต่อสารสกัดจากยีสต์ 100 มิลลิลิตรที่ปริมาณของแข็ง 5 %

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LIST OF ABBREVIATIONS

ca	About, approximately
°C	Degree of Celsius
cm	Centimeter
ed.	Edition
e.g.	Exempli gratia (Latin), for example
et al.	Et alii (Latin), and others
g	Gram
hr	Hour
i.e.	Id est (Latin), that is
kg	kilogram
kW-h	kilowatt-ohm
L	Liter
µ	Micron
µg	Microgram
mg	Milligram
mL	Milliliter
mm	Millimeter
min	Minute
M	Molarity
MWCO	Molecular weight cut off

LIST OF ABBREVIATIONS

(continued)

m^3	Cubic meter
nm	Nanometer
No.	Number
/	Per
%	Percent
UV	Ultraviolet
Vol.	volume
w/v	Weight by volume
w/w	Weight by weight

CHAPTER I

INTRODUCTION

During the last 30 years, there has been a rapid growth in demand for savory flavor. While the supply of beef extract or whale extract is limited because of its high cost, yeast extract can be easily prepared from baker's yeast and brewer's yeast in large quantities at a low price because their flavor closely of meat extract [1]. They were widely used as a component of various seasonings [2].

In general, since the surplus yeast in the brewing industry is relatively cheap, it is utilized extensively in the production of yeast extract. However, brewer's yeast is likely to contain undesirable flavor characteristic [3] thus debittering is considered as an important further step. According to a conventional method, yeast extracts can be prepared by autolysis, or by hydrolysis using enzyme, acid or alkali. Among these methods, autolysis is the most preferred method since the quality of the resulting yeast extract is excellent.

It has been known that the quality of the yeast extract, especially thickness or body in taste, can be improved by addition of disodium guanosine-5′-monophosphate (5′-GMP) or disodium inosine-5′-monophosphate (5′-IMP) which is known to be a flavor enhancer contained in shiitake mushroom or dried skipjack [4]. These flavor enhancers have been intensively studied their property and found that they can improve the intensity, mouthful and continuity of other food systems. And it has been well known that a considerable amount of RNA is contained in yeast cell. Since

nucletides are the builing blocks of ribonucleic acid, it is logical to use yeast RNA for the production of this flavor enhancing 5′-nucleotides [5].

According to a conventional method, a yeast extract containing flavoring 5′-nucleotides is prepared by extracting an intracellular RNA from yeast cells by heating a suspension of yeast cells, hydrolysing the extracted RNA with 5′-phosphodiesterase into 5′-nucleotides containing GMP, and adding the resulting flavoring 5′-nucleotides to an acid or enzyme-hydrolysed solution of residual yeast cells from which RNA is previously extracted [4]. However, this conventional method is not economical since an expensive enzyme has to be used.

Special enzymes derived from a microorganism belonging to a ray fungus have been used as a source of enzyme to produce flavoring 5′-nucleotides in the autolysis process. But this process can not be carried out practically since the safety of the enzyme and microorganism used for producing the enzyme are not legally approved [4]. Malt rootlets have been reported that they contained the 5′-phosphodiesterase [6]. They were used as the one of interesting source of enzyme for producing yeast extract containing 5′-GMP because they can be easily available, cheap and also no doubt about safety of enzyme.

Nevertheless, the study of producing yeast extract containing 5′-GMP previously mentioned was mostly studied base on the local raw material of their country. In Thailand, the field of this study is relatively unintensive.

It is, therefore, a principle objective of this study is to investigate the possibility to prepare yeast extract containing 5′-GMP from brewer's yeast derived from the beer industry in Thailand as well as malt rootlets, a by product from malting industry in Thailand, which is used as a source of the enzyme 5′-phosohodiesterase.

CHAPTER II

LITERATURE REVIEW

1. Food additive

Food additive may be defined as any substance that becomes part of the final food product whether added intentionally or incorporated accidentally [7]. From a regulatory standpoint, each of the food additives must provide some useful and acceptable function or attribute to justify its usage. Generally, improved keeping quality, enhanced nutritional value, functional property provision and improvement, processing facilitation and enhanced consumer acceptance are considered acceptable functions for food additives [8].

Yeast extract is also one kind of food additive. They are commonly used instead of meat extract for palpability improvement of foods.

2. Yeast extracts

During the last 30 years, there has been a rapid growth in demand for savory flavors for use in formulated foods, and the use of meat extracts for such applications has failed to remain competitive because of its high cost. To overcome this problem, researchers began to investigate the use of yeast for the production of extracts as suitable replacements for meat extract.

Yeast extract is a concentrate of soluble material derived from yeast following hydrolysis of the cell material, particularly the proteins, carbohydrates and nucleic acids. This is generally carried out by use of its own hydrolytic enzymes

(autolysis) or by other methods (plasmolysis or hydrolysis) in order to release the cell content in a highly degraded form [9,10]. Yeast extracts have flavor strength substantially higher than that of dried yeast on as equal solids basis because of the concentration effect in extracts following the separation of the cell wall fraction during process [11].

Yeast extracts have a flavor closely resembling that of meat extract. They are used in all applications that require the addition or enhancement of a meaty flavor which are utilized in numerous processed and convenience foods where a meaty flavor is sought such as dried soups, gravy granules, flavor potato snacks, 'Marmite' and 'vegemite' and also for their ability to promote growth of microorganism in industrial fermentation [2]. Yeast extracts are commercially available as a powder or paste and have been used extensively by the food industry as a flavoring agent. They are also cost- effective in relation to other flavoring agents on the basic of equivalent flavor intensity.

3. Source of yeast using as raw material

The key factors in choosing the starting material for producing yeast extract are the price, availability of the yeast itself and the desired properties such as flavor profile, color, etc. of the final products.

The raw material for the manufacture of yeast extract is either the primary-grown yeast such as *Saccharomyces cerevisiae* (baker's yeast), *Candida utilis*, *Kluyveromyces marxianus* (formerly *S. fragilis*), or the secondary yeast such as *Saccharomyces uvarum* (formerly *S. carlbergensis*) [12,13].

3.1 Spent brewer's yeast

Spent brewer's yeast (*Saccharomyces uvarum*) is the by-product of beer manufacturing. It is a cream or slurry containing about 15-20 % total solids of which approximately two-thirds are yeast solids and one-third are beer solids. Spent brewer's yeast however has not been easy to market [14]. Waste yeast may be sold in the paste form or dried in drum dryers for inclusion in animal feed. Other means of disposal for the spent yeast was by spraying it on agricultural lands as a source of fertilizer. The yeast may also be used directly as a source of vitamin [15].

Spent brewer's yeast can be used as an important by-product. Its nutritional and biological value as well as chemical and physical properties open up wide possibilities of application [16] such as the high protein and vitamin contents of dried brewer's yeast make them attractive as natural supplements for improving the nutritional profile of human food and animal feed. Spent brewer's yeast is interesting for choosing as a raw material for producing yeast extract due to its cost less than primary grown yeast. However, brewer's yeast tends to produce lower yields and darker colored extracts with characteristically different flavor profiles [11]. In addition, it has a strong bitter flavor. It is usually necessary to debitter the yeast or remove adsorbed hop bitter substances before using.

3.2 Debittering of spent brewer's yeast

Brewer's yeast which used as raw material for yeast extract production is likely to contain undesirable flavor characteristics as a result of carry over of hop resins and beer solids from the brewery fermentation. All beers have the property that the flavor is mainly derived from hops. The essential components are the so-called hop

α - acids or iso-humulone [17]. These α-acids are tasteless, but upon prolonged boiling they are isomerized to the very bitter-tasting iso-α-acids or iso-humulone [18]. The iso-α-acids associate with the surface of the yeast cells present rendering brewer's yeast intensely bitter [19].

As the spent brewer's yeast has a strong bitter flavor, it is usually necessary to debitter before use although the treatment able reduces the final extract yield [3]. The bitter substances are commonly removed by an alkaline wash process [20,21,22]. Methods to remove bitter substance from spent brewer's yeast using an alkaline wash, 2% sodium carbonate or 1 % ammonium carbonate solution, combine with water wash [21] or by urea, thiourea and potassium thiocyanate solution, have been reported [20]. Most reports have focused on alkaline washed because of its cost. Few extract manufactures rely on ion exchange technology [23].

A patent reported about a debittering process of brewer's yeast and brewer's yeast autolysate by contacting a yeast suspension or yeast autolysate with a composite absorbent and magnetic particle embedded in a porous matrix of organic polymeric material [24]. Alternatively, activated carbon and solvent extraction have been used to remove bitter substance from product. However, the bitterness level of the final extract can be controlled by the extent to which spent yeast is clean prior to autolysis.

4. Production of yeast extract

Technically, extracts from yeast can be produced in various ways. The processes differ both in their basic procedures and in the functional properties of the

products obtained. There are several distinct processes for yeast extract production; autolysis, plasmolysis, hydrolysis and mechanical distruption.

4.1 Autolysis

Autolysis (self-destruction) is a process by which the cell components are solubilized by activation of the degradative enzymes inherently present within the cell. It is achieved by the application of carefully controlled conditions such as temperature, pH, time and addition of certain enhancing agents under conditions which will kill the yeast population without inactivation the degrading enzymes that remain compartmentalized in live cells [3,10].

As the cells die under these conditions, disorderliness occurs within the cells, allowing the free degradative enzymes to indiscriminately attack their specific substrates. This causes the breakdown of the corresponding macromolecules like proteins and nucleic acids to their basic units. Disorderliness within the cell wall and a loss in the integrity of the semi-permeable membranes. It eventually permits the soluble components to leak out of the cell into the surrounding environment. The cell wall which is primarily made up of glucan and mannan, which are high molecular weight polysaccharides, subsequently becomes porous and allows ready passage of soluble components from within the cell [25]. Different stages of yeast autolysis are shown in Figure 1.

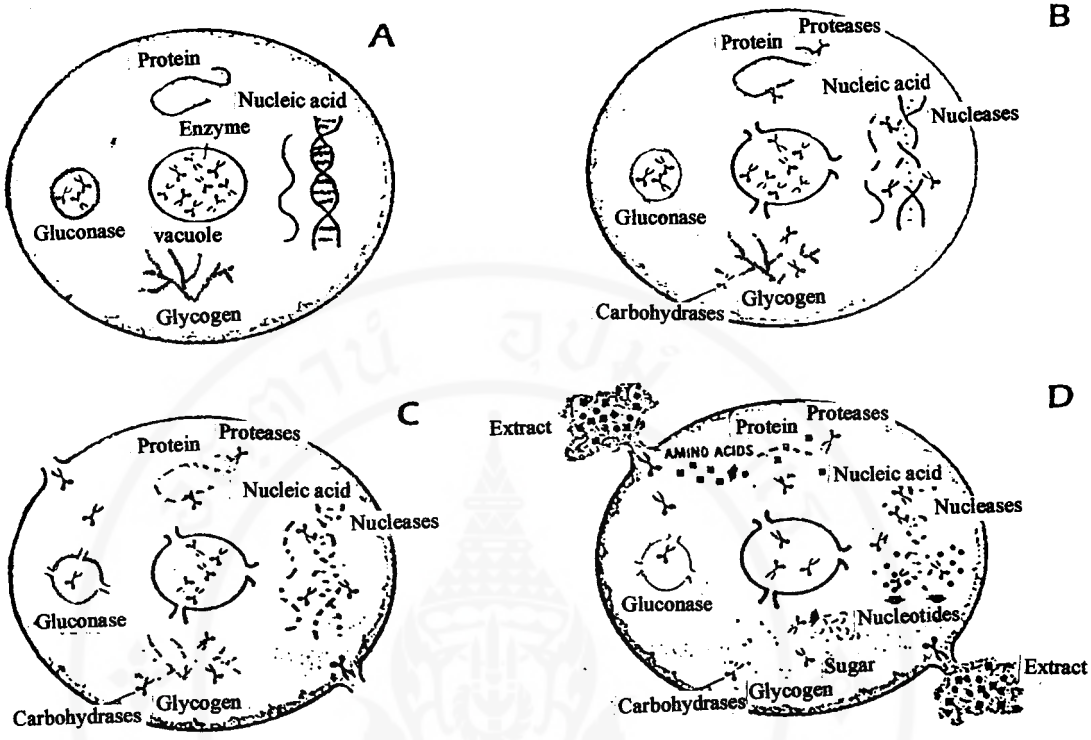


Figure 1 Different stages during autolysis of yeast [9]

4.2 Autolysis procedure

The autolysis procedure is well suited for the production of low-sodium yeast extracts that wide acceptance in the food industry [3]. In a typical autolysis, a slurry or cream of live yeast cells at 15-20 percent solids is plasmolyzed with 2-5 weight percent salt (based on cell weight). The slurry is heated up to 45-60 °C and held at that temperature for 12-36 hr, or until the desired degree of solubilization is reached at pH approximately 5.0-5.5. The resulting autolyzed yeast materials is then pasteurized at 80-100°C, cooled and centrifuged or filtered with diatomaceous earth. The filtrate may be concentrated to a paste of about 70-80 weight percent solids, or spray dried to give a hygroscopic powder containing about 3 to 5% moisture [1,26,27]. The commercial production processes of yeast extract are shown in Figure 2.

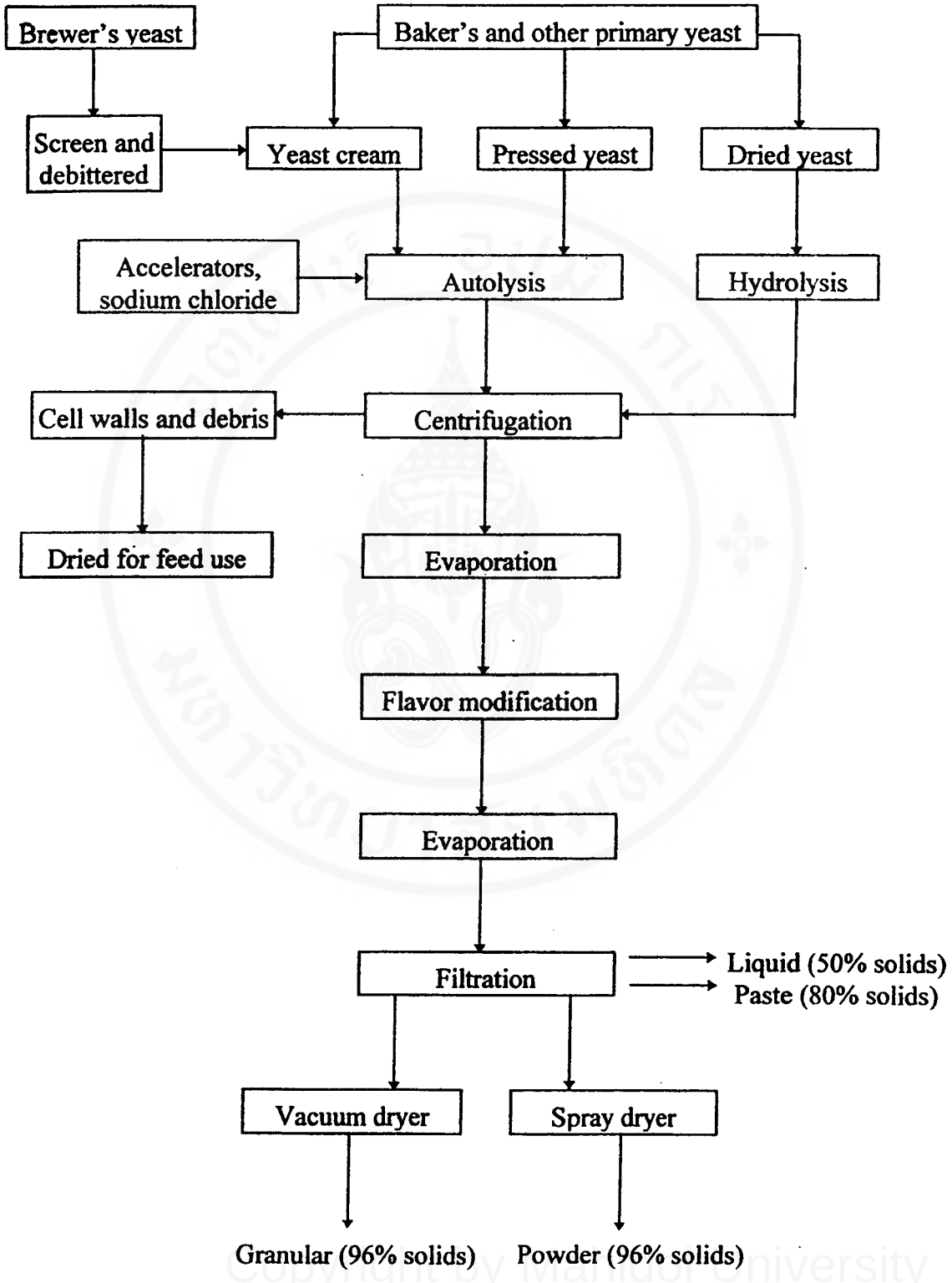


Figure 2 Process routes for the commercial production of yeast extract [28]

5. Flavor enhancers

Numerous additives, mostly natural, are being used to modify or enhance the sensory properties of different foods. This fact is the basis for the additive of spices and flavoring compounds to foods to improve palatability. However, the term flavor enhancer or flavor potentiator has been reserved for a few selected compounds which have very little taste of their own but have the ability to significantly influence the taste perception of others such as the basic savory attributes of certain types of foods in order that these foods become organoleptically attractive to the human palate.

The best known flavor enhancers that are in commercial use, worldwide, are monosodium glutamate (MSG), inosine 5′- monophosphate (5′-IMP), and guanosine 5′-monophosphate (5′-GMP). The later two compounds are sometimes referred to as disodium 5′-inosine or disodium 5′-guanylate, respectively.

The discovery of flavor enhancers was started in 1909. The functional flavor ingredient present in sea tangle which was otherwise referred to as Kombu in Japanese (*Laminaria japonica*) was identified as monosodium glutamate [29]. In the 1960s, the active flavor enhancing component of black mushroom, shiitake (*Lentinus edodus*) was identified as guanosine 5′-monophosphate (5′-GMP) [30].

Although MSG and 5′-nucleotides were originally isolated from natural sources, they were found too costly to be produced by this approach for commercial purposed. Subsequent research in this area made it possible to produce these products at a lower cost on a commercial scale through the use of fermentation technology. They are now generally known to accentuate the savory and meaty flavor of foods and have found application in soups, gravies, and many other savory products.

5.1 Umami taste

Much of the current research in taste is based on the concept of four basic tastes: sweet, sour, salty, and bitter. However, the lines of evidence presented by more recent studies suggest that a few types of tastes cannot be completely analyzed and described in terms of these four basic tastes. For example, 5′-ribonucleotide (like 5′-GMP and 5′-IMP) and MSG have been reported to have a distinct savory and delicious taste in certain foods where the taste realm extends beyond the four basic tastes. This uniquely different taste is now referred to as *umami*, which is derived from the Japanese word meaning *savory* or *delicious* [29].

Experimental data on receptor mechanisms of umami substances in animals have shown that receptor sites for umami are independent of that specific for the four basic tastes. These data suggest that the range of taste is broader and the taste of umami may therefore be considered as another basic taste. However, the question of whether umami represents an independent and uniquely different entity of oral sensation is not resolved yet [31-33]. The flavor-enhancing effect of umami substances on the palatability of different types of foods has been extensively investigated [34].

5.2 Taste synergism

In the 1960s, Kuninaka [35] observed the remarkable synergism that exists between L-glutamate and the 5′-ribonucleotides; 5′-GMP and 5′-IMP. This type of synergism, not common among other taste stimuli, is one of the most remarkable properties of these flavor potentiators. Synergism is usually evaluated by examining whether or not the response to a mixture of two components is greater than the sum of the two individual responses. A well-know sensory synergism between MSG and

some ribonucleotides, observed at the receptor level in the catfish, occurs because of increased binding of molecules [36]. Although the receptor mechanism of the synergism between MSG and 5′-nucleotides is still unknown, several hypotheses have been proposed [33,35,37-40]. According to one hypothesis [41], the receptor for glutamate shows an allosteric transition due to the binding of 5′-nucleotides (5′-GMP or 5′-IMP) to its regulatory subunit. The conformational change that is elicited by this binding results in the exposure of additional glutamate binding sites of the receptor proteins, thereby enhancing the taste response.

Several combination of MSG and 5′-GMP and/or 5′-IMP are now commercially available for use in the food industry. Most popular blends have a MSG: 5′-nucleotide ratio in the neighborhood of 95:5. Because of the improved efficacy of these blends due to the phenomenon of synergism as just eluded to, these flavor enhancers will be used at relatively low concentration in food formulations thereby providing a cost advantage to the food processor.

The low concentrations of 5′-nucleotides are capable of providing an impression of a much greater concentration of glutamate than is actually present in the food system. For example, the threshold values of 5′-IMP and 5′-GMP are 0.025 and 0.0125% respectively, when tasted individually. The corresponding value of a 50:50 blend of 5′-GMP and 5′-IMP has been reported to be 0.0063 %. When used in combination with 0.8 % MSG, the resultant taste threshold decreased to 0.00013 % representing a dramatic reduction of the taste threshold due to the strong synergism that comes into play between these flavor enhancing compounds [42].

6. 5′-Nucleotides

6.1 Structure of 5′-nucleotides

Several researchers have been able to elucidate the relationship between flavor potentiation and molecular structure of different types of nucleotides [43-47]. Of the three isomeric forms (2′, 3′and 5′) of nucleotides (Figure 3) that exist in foods, only some of the 5′-nucleotides are known to exhibit the flavor enhancing properties.

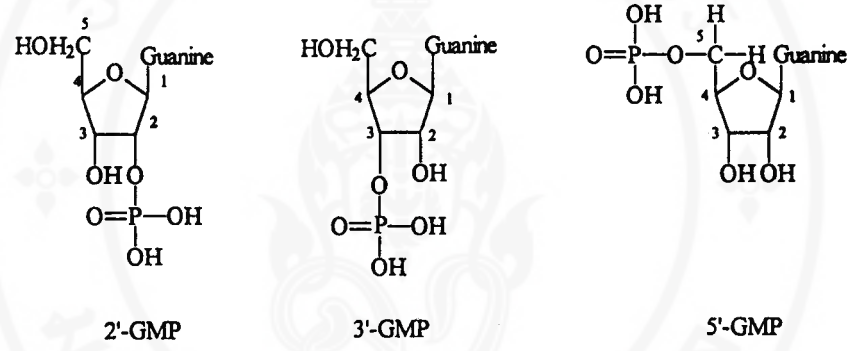


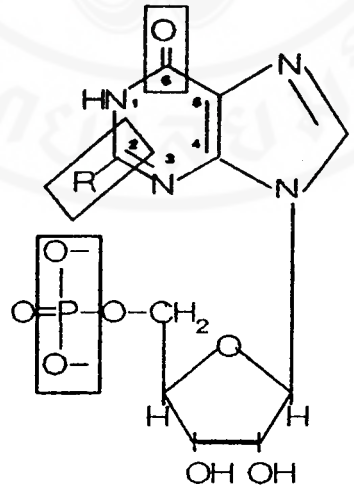
Figure 3 Three isomers of GMP [42]

The flavor nucleotides recognized as having umami taste are those nucleotides that have the purine base with hydroxyl groups (or keto, its tautomeric form) at the 6-position and a ribose moiety esterified with phosphoric acid at the 5-position. Additionally, the hydrogen atom at the 2-position in the purine base could be replaced by the hydroxyl (Xanthosine 5′-monophosphate or 5′-XMP) or amino group (5′-GMP) without seriously affecting the flavor enhancing properties.

However, it is important to note that 5′-XMP which has OH groups at C-2 of the purine ring has the weakest taste intensity of the three naturally occurring flavor nucleotides. Hence it is the least important nucleotide of the three, from a

commercial standpoint. Of the other two (5′-GMP and 5′-IMP), 5′-GMP is generally perceived as having a higher flavor enhancing intensity than 5′-IMP [1].

In addition, Honjietal [48] demonstrated that the two OH groups on the phosphorus of the 5′-nucleotide molecule are also critical for efficient flavor potentiation. The order of effectiveness of 5′-nucleotides in human is GMP > IMP > XMP and adenosine monophosphate is usually judged ineffective. The pyrimidine nucleotides are also generally known to be ineffective. More elaborate studies by Kuninaka [49] on taste reception have shown that each species of flavor enhancing 5′-nucleotide has at least three receptor sites in the receptor domain for attachment. These three critical groups are present in purine-based 5′-nucleotides, which as the author claims, are the points of attachment to the three sites on the receptor domain as shown in Figure 4.



GMP: R represents NH_2; IMP: R represents H; XMP: R represents OH

Figure 4 General structure of 5′-purine nucleotide showing the groups that are complementary with the three sites on the receptor demain.

6.2 Industrial production of 5′-nucleotides

In the 1950s, production techniques that involve microbial fermentation with a higher level of productivity came into widespread use for both 5′-inosinic acid and 5′-guanylic acids. Today, several processes for the production of these flavor enhancers are being used and the most widely used methods are listed below [23,40,50-52]:

A) Direct fermentation of sugars into 5′-GMP and 5′-IMP.

B) Direct fermentation into nucleosides with subsequent phosphorylation into corresponding 5′-nucleotides.

C) Degradation of microbial RNA to 5′-nucleotides using 5′-phosphodiesterase enzyme. Subsequent conversion of 5′-AMP to 5′-IMP with adenlic deaminase enzyme.

D) Any combination of the above three procedures.

Almost the entire supply of high purity 5′-GMP and 5′-IMP available today is commercially produced by applying the direct fermentation techniques [53].

7. Production of 5′-nucleotide-rich yeast extracts

7.1 5′-phosphodiesterase

5′-phosphodiesterase (PDE, orthophosphoric diester phosphohydrolase, EC 3.1.4.1) is wide spread in animal, plant and microorganism. Those enzymes from snake venom [54], pig kidney [55], rat liver [56], calf kidney [57], *Aspergillus niger* and *Flammulina velutipes* have been studied by several groups. Since 1966, PDE from plants such as carrot [58,59], tobacco cell [60], oat and sugar beet leaves has been purified and characterized.

PDE from snake venom and some microorganisms has been utilized for the characterization of oligonucleotides and as a tool in sequence analysis. Microbial PDEs had also been used in the degradation of RNA for the production of the seasoning 5′-nucleotides for commercial interests. Barley rootlet mass-procuded as a by-product in brewing industry is a rich source of RNA-degrading enzyme. Benaiges *et al,* 1981 partially purified the 5′-phosphodiesterase from barley rootlets for the degradation of RNA to obtain 5′-nucleotides [61].

7.2 Commercial production of 5′-nucleotide-rich yeast extracts

Yeast is generally known for its high RNA content (2.5-15%) [5]. Since nucleotides are the building blocks of ribonucleic acid, it is logical to use yeast RNA for the production of these flavor enhancing 5′-nucleotides. Yeast is generally recognized as safe (GRAS) and can be grown economically to produce large quantities of biomass rich in ribonucleic acid. The RNA content is highly strain dependent. Among yeasts, *Candida utilis* generally contains the highest RNA content (10-15%) on a dry solid basis. Baker's yeast strains (*S.cerevisiae*) are generally lower in RNA content (8-11%) but can be used economically for the production of 5′-nucleotide-containing extracts. To maximize the RNA content, it is necessary to harvest the yeast in the logarithmic phase when protein synthesis is at its peak.

The hydrolysis process for the commercial production of 5′-nucleotide-rich extracts consists of two important step (Figure 5) [1]. The critical first step consists of a treatment that released RNA from the yeast cell into the surrounding medium. The second step is an enzymatic treatment that converts RNA to 5′-nucleotides.

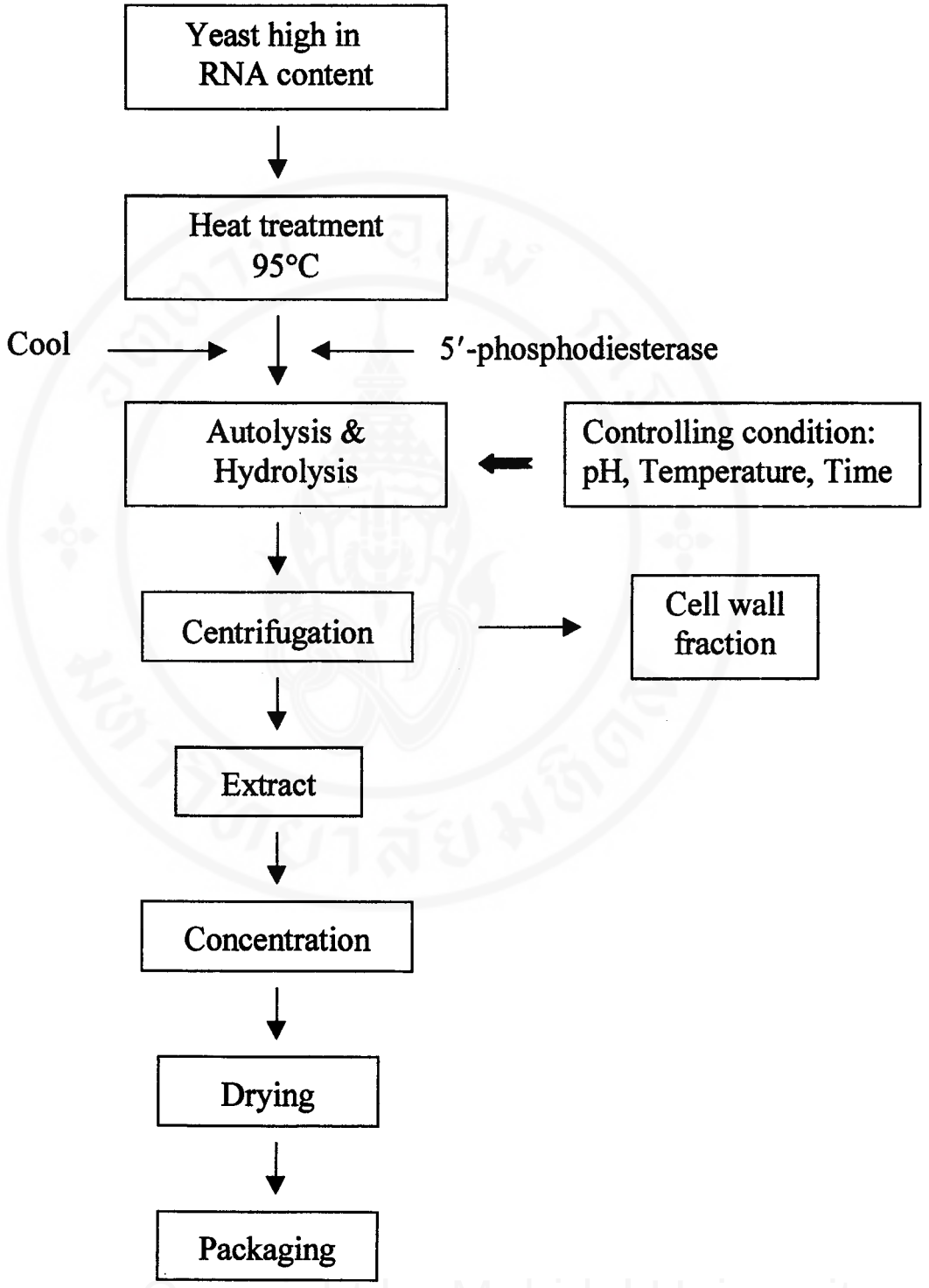


Figure 5 Flowchart for the commercial production of 5′-nucleotide-rich yeast extracts [1]

Most of the RNA in the cell is extracted by heating the yeast suspension to 90 to 100°C for 1 to 3 hr. By controlling the pH of the medium in the 6.0 to 6.6 range, it is possible to maintain the undesirable nucleases in the unreactive form prior to their complete destruction at high temperature. These nucleases convert RNA to 2′- and 3′- nucleotides, which unlike the 5′-nucleotides have no effect on flavor in food systems. Subsequent heat treatment causes the destruction of all enzymes and the release of soluble low-molecular-weight components from the cell.

The second phase of the process starts with cooling of the RNA-rich medium to 50 to 60°C at pH 6.5; this is followed by the initiation of the RNA hydrolysis using the enzyme 5′-phophodiesterase. This and other 5′-forming enzymes which are found in yeast at low concentrations are generally masked by the other more active nucleases that degrade nucleic acids to nucleotides other than 5′-nucleotides. For this reason, the 5′-phosphodiesterase generally must come from an external food-grade source. Some commercially available enzymes are derived from certain fungi such as *Penicillium citrinum* or certain *Actinomyces* such as *Streptomyces aureus*.

However, these organisms are not considered GRAS in the United States, so that enzymes derived from them cannot be used [4]. As an alternative, the presence of 5′-phosphodiesterase activity in cereal germs has been demonstrated by Schuster [62]. Also, malt rootlets (which are considered GRAS) are by-products in the malting industry and are now serving as a reliable source of 5′-phophodiesterase activity.

Following the enzymatic hydrolysis, the yeast extract contain four types of 5′-nucleotides, namely, 5′-guanosine monophophate (5′-GMP), 5′-uridine monophosphate (5′-UMP), 5′-cytidine monophosphate (5′-CMP), and 5′-adenine monophophate (5′-AMP), corresponding to the four bases present in RNA as shown in

Figure 6. Of these four 5′-nucleotides, only 5′-GMP provides the flavor-potentiating property to the extract.

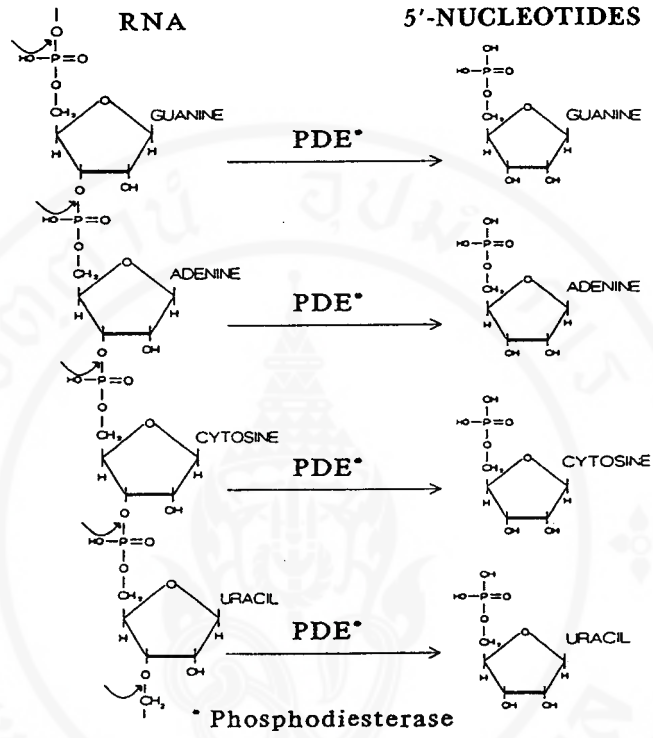


Figure 6 Degradation pattern of RNA in the presence of the enzyme 5′-phosphodiesterase

Although 5′-AMP offers on flavor enhancing property, it can serve as a precursor of the flavor enhancing compound 5′-IMP (Figure 7) [63]. Crude extracts rich in 5′-IMP along with 5′-GMP are currently produced commercially by use of the two enzymes, 5′-phosohodiesterase and adenylic deaminase, acting in sequence [64]. These yeast-derived products have both flavor-enhancing properties and background flavor. The two remaining 5′-nucleotides (5′-CMP and 5′-UMP) do not possess flavor enhancing properties but have found use in the pharmaceutical industry for the production of antiviral drugs [65].

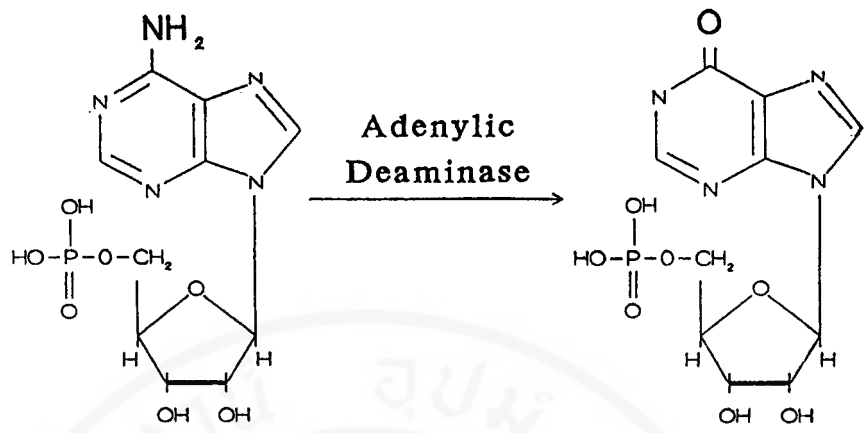


Figure 7 Conversion of 5′-AMP to 5′-IMP by the action of the enzyme adenylic deaminase

Researchers have developed genetically modified edible yeast strains capable of producing 5′-nucleotide-rich yeast extracts during autolysis without the addition of 5′-phosphodiesterase [66-67]. In these strains the endogenous nucleases mediate the degradation of RNA to preferentially form 5′-nucleotides instead of the basis of 3′-nucleotides at pH 8.5 to 9.5 and 40 to 45°C. These mutants are selected on the basis of their sensitivity to 5-fluorouracil (5-FU); however, they are generally found to be slow growers. Because of this serious deficiency, the approach has so far met with limited success.

7.3 5′-Nucleotidase

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) was discovered by Reis in 1934 [68]. The enzyme specifically catalyses the dephosphorylation of nucleoside phosphates having phosphate groups attached at the C-5 position of the ribose ring

$$\text{Adenosine-5′-monophosphate} + H_2O \xrightarrow{\text{5′-Nucleotidase}} \text{Adenosine} + H_3PO_4 \text{ [68]}$$

5′-Nucleotidase was isolated from various sources. For example; bell semen, microsomal fraction and human placenta [69]. It was also found in rapidly proliferating parts of germinating seeds, such as the rootlets and stems. From the 5′-nucleotidase property makes the crude extract from the rootlets or stems unsuitable for use in preparing 5′-nucleotides from RNA [70]. The method for preparing an aqueous enzyme medium capable of forming 5′-nucleotides from RNA was disclosed. This method the seed parts are heated in water to form about 70°C to 85°C for several minutes [71,72]. The other preparing methods were the enzyme solution obtained by extracting broken malt roots with water and then heating the clear filtrate at a temperature of 60°C to 65°C for 5 to 10 min [4]. Heating the extracted enzyme at a temperature of 60-65°C for 15 min to 1 hr [70], 45-70°C for 5-40 min [73] and addition of inhibitor (Zn Ac_2. H_2O 0.001-0.01 M) [74] were also reported.

8. Regulatory and Safety of Flavor and Flavor Enhancers

The Food and Drug Administration (FDA) believes that some segment of the population (approximately 1-2 % according to certain surveys) experience a mild and transitory reaction under certain circumstances when they consume MSG at levels such as found in heavily-enhanced foods. The alleged symptoms include skin flushing, tightening of jaws and upper chest muscles, burning, headache, nausea etc, which fall under the catch all term "Chinese restaurant syndrome" [75].

In 1958, the Food Additive Amendment to the Federal, Food, Drug and Cosmetic Act (FFD&C) designated L-glutamic acid, hydrolyzed protein products containing L-glutamic acid (HVP and yeast extract) as Generally Regarded as Safe (GRAS). On June 21, 1991, the FDA published a proposal to not require listing MSG

on a product label when protein hydrolysates are added to food products (Office of the Fedral Register 1991). Currently, MSG remains in the GRAS list with a requirement that it must be identified as "monosodium glutamate" on the ingredient label of any food to which it is added. Yeast extracts meet the definition of "natural flavoring" although the U.S. Department of Agriculture requires the use of the common or usual name "yeast extract" on the label. Both 5′-IMP and 5′-GMP are also approved for use in food applications by the FDA and must be labelled by their common names.

8.1 MSG

Approximate daily dietary intakes of total and free glutamate levels by human adults are 23 and 0.7 g, respectively. This amounts to approximately 0.34 g of total glutamate consumption/kg body weight by humans on a daily basis. Monosodium glutamate, as added to foods, provides a daily dietary intake of 0.55 g which is equivalent to about 0.008g/kg body weight for humans [76]. In western as well as eastern countries, per capita daily intake of added MSG remains higher in eastern regions of the world (4g at the 97.5th percentile in Korea) than in the west (less than 1 g). In the USA, the mean daily intake of MSG per capita has been estimated at 550 mg [77]. However, large variations exist around these mean values. For example, 'extreme consumer' with a daily ingestion of 2.3 g have been indentified in the UK [78].

8.2 Flavor enhancing 5′-nucleotides

Both 5′-IMP and 5′-GMP are approved for use as food ingredients by the FDA and must be labelled by their common names. However, yeast extracts containing 5′-GMP and 5′-IMP meet the definition of "natural flavors". Yeast extract

containing low levels of flavor enhancing 5′-nucleotides can, however, be labelled as "yeast extracts". Dietary nucleotide, once thought unnecessary for normal physiological functions have now begun to show their effectiveness in infant nutrition, adult cellular immune functions, intestinal development and repair and other stress related phenomena both in young and old. It is now being incorporated even in geriatric foods. Evolutionary processes have made human milk the carrier of the most optimal profile of nucleic acid and nucleotides for optimal infant development.

The scientific information on the safety of 5′-nucleotides has been derived mainly from the work of Kojima (1974). Much of the adverse effects reported in mice and rats in this study have occurred only after intravenous administration of mega doses of 5′-IMP and 5′-GMP into these test animals. Therefore, there is a dearth of information on the effect of orally ingested 5′-nucleotides. However, considering the fact that intravenously administered mega doses of 5′-nucleotides are needed to initiate any adverse effect in rats and mice, it is difficult to visualize any toxic symptoms in such test animals ingestion significantly lower levels of 5′-nucleotide orally. There are certain yeast extracts that contain 2-6 % natural flavor enhancing 5′-nucleotides which are primarily 5′-GMP and 5′-IMP. When this product is included in 8 oz of soup, a maximum of 70 mg of both 5′-GMP and 5′- IMP can be expected, when used at 0.5 % in the final product. This amount of 5′-nucleotides is significantly lower than the estimated daily nucleotide need of 480 mg for a human infant [79].

9. Enzyme concentration by precipitation

Precipitation is usually only used as a fairly crude separation step often during the early stages of a purification procedure, and this is then followed by

chromatographic separations. Precipitation can also be used as a method of concentrating proteins prior to analysis or a subsequent purification step.

9.1 Precipitation by increasing the ionic strength (salting-out)

Precipitation by addition of neutral salts is probably the most commonly used method for fractionating proteins by precipitation. The precipitated protein is usually not denatured and activity is recovered upon redissolving the pellet. In addition these salts can stabilize proteins against denaturation, proteolysis or bacterial contamination. Thus, a salting-out step is an ideal step at which to store an extract overnight, either before or after centrifugation. The cause of precipitation is different from that for isoelectric precipitation, and therefore the two are often used sequentially to obtain differential purification. Salting-out is dependent on the hydrophobic nature of the surface of the protein. Hydrophobic groups predominate in the interior of the protein, but some are located at the surface, often in patches. Water is forced into contact with these groups, and in so doing becomes ordered (Figure 8).

When salts are added to the system water solvates the salt ions and as the salt concentration increases water is removed from around the protein, eventually exposing the hydrophobic patches. Hydrophobic patches on one protein molecule can interact with those on another, resulting in aggregation. Thus, proteins with larger or more hydrophobic patches will aggregate and precipitate before those with smaller or fewer patches, resulting in fractionation. The aggregates formed are a mixture of several proteins, and like isoelectric precipitation the nature of the extract will affect the concentration of salt required to precipitate the protein of interest. In contract to isoelectric precipitation, increasing the temperature increased the amount of

precipitation; however, salting-out is usually performed at 4°C to decrease the risk of inactivation (by, e.g. proteases) [80].

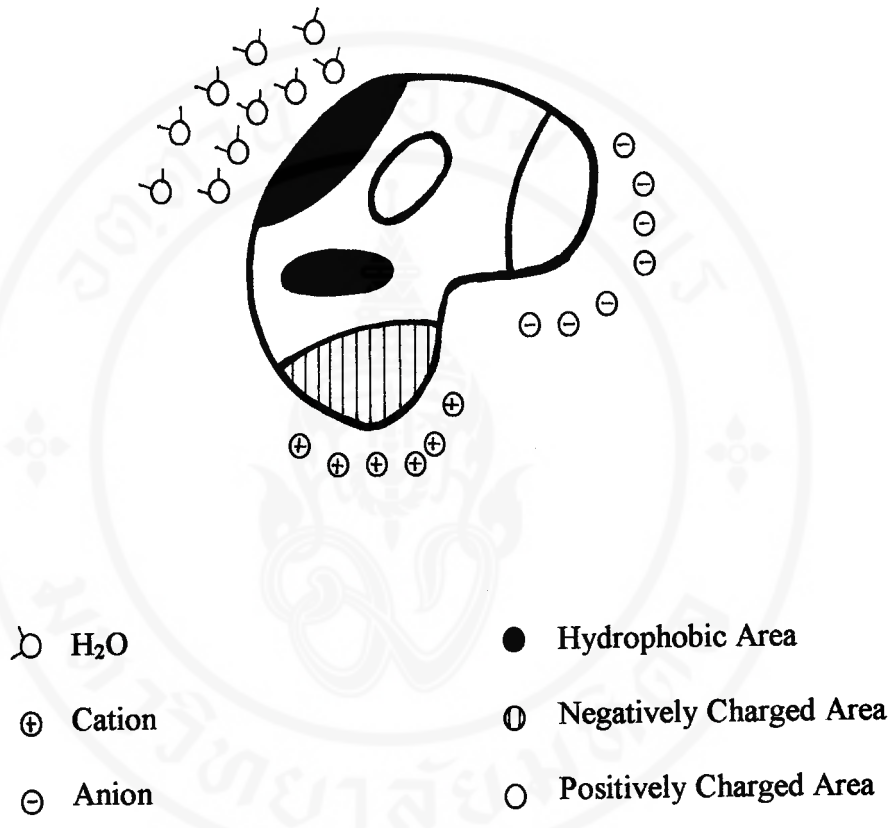


Figure 8 Schematic representation of a protein showing negatively and positively charged areas on the protein interacting with ions in the solution. The hydrophobic areas on the protein interact with water molecules causing an ordered matrix of water molecules to form over these areas.

In pactice ammonium sulphate is the most commonly used salt (other salts which have been used in particular applications are ammonium acetate, sodium sulphate, and sodium citrate). Ammonium sulphate is cheap, and sufficiently soluble.

9.2 Dialysis

Frequently it is necessary to remove salts or change the buffer after one step in the purification for the next step to work efficiently. This is often achieved by dialysis; the protein solution is placed in the required buffer, small molecules can pass freely across the membrane whilst large molecules are retained. The semi-permeable dialysis tubing is usually made of cellulose acetate, with pores of between 1-20 nm in diameter. The size of these pores determine the minimum molecular weight of molecules which will be retained by the membrane (MWCO). Dialysis tubing often requires pre-treatment to ensure a more uniform pore size and removal of heavy metal contaminants. Equilibrium is usually reached after approximately 3 hour with efficient stirring and 15,000 MWCO membranes; the time taken increases with decreasing MWCO. Dialysis is often carried out overnight, usually at 4°C to minimize losses in activity [80].

CHAPTER III

MATERIALS AND METHODS

1. Spent brewer's yeast

Yeast used in experiments was spent brewer's yeast generated as a by-product from beer production. It was kindly provided as a slurry by Boon Rawd Brewery Co., Ltd. (Samsen plant), Bangkok, Thailand.

2. Rootlets

2.1 Dried malt rootlets

Dried malt rootlets were obtained as a by-product of malting industry. They were kindly provided by Chiangmai Malting Co., Ltd. Sarapee, Chiang Mai, Thailand. The appearance of dried malt rootlets were small pieces of broken dried rootlets with a length roundly 2-3 mm and brown color as shown in Figure 9.

2.2 Fresh malt and rice rootlets

Barley was kindly provided by Chiangmai Malting Co., Ltd. Sarapee, Chian Mai, Thailand. Rough rice was purchased from local market. The barley and rice grains were cultivated in laboratory and their rootlets were harvested for used as raw material in the experiments.

Figure 9 Dried malt rootlets

3. Chemicals

Potassium dihydrogen phosphate (KH_2PO_4), potassium hydrogen phosphate (K_2HPO_4), acetonitrile (CH_3CN) HPLC grade, magnesium chloride ($MgCl_2$), Tris [hydroxy methyl] amino methane ($C_4H_{11}NO_3$), hydrochloric acid (HCl), perchloric acid ($HClO_4$), sodium hydroxide (NaOH), ethylene diamine tetra acetic acid (EDTA), bovine serum albumin (BSA), copper sulphate ($CuSO_4.5H_2O$), sodium potassium-tartrat ($C_4H_4KNaO_6.4H_2O$), sodium carbonate (Na_2CO_3), folin-ciocalteau reagent, ammonium sulfate ($(NH_4)_2SO_4$), sodium azide (NaN_3), clorox, chloroform, zinc acetate ($ZnAc_2.H_2O$) and calibration buffer solution of pH 4, 7 and 10 were products of Merck (Germany). All reagents were analytical grade.

Guanosine 5′-monophosphate (5′-GMP) disodium salt (minimum 99%: from yeast), inosine 5′-monophosphate (5′-IMP) disodium salt (grade III: from yeast), uridine 5′-monophosphate (5′-UMP) disodium salt (from yeast), adenosine 5′-monophosphate (5′-AMP) disodium salt (from yeast 99+%), guanosine 2′-monophosphate (2′-GMP) disodium salt (from yeast), guanosine 3′-monophosphate (3′-GMP) disodium salt (from yeast), thymidine 5′-monophosphate p-nitrophenyl ester (nitrophenyl-pdT) and ribonucleic acid (RNA) standard from baker's yeast. They were purchased from Sigma Chemicals Company, Missouri, USA . Methanol (HPLC grade) was purchased from J.T.Baker, Solusorb, USA. Uracil and acenapthene were purchased from Waters Co., Ltd, Milford, Massachusetts, USA.

Dialysis tubing (Cell–Sep T3 MWCO: 12,000–14,000) was purchased from Membrane Filtration Products, Inc., San Antonio, USA. Nylon membrane filter (pore size 0.25 μm) was purchased from Lida Manufacturing corp., Rochester, New york, USA and Nylon membrane filter (pore size 0.45 μm) was purchased from Gelman Sciences Inc., MI, USA.

4. Equipment

The equipment used in this study included a Autoclave (ss320, Tomy Seiko, Tokyo, Japan), Blender (Moulinex model x 13, France), Centrifuges (Mode Himac CR 20B2, Hitachi, Japan and Beckman J-6M/E, USA and Sorvall RC 3B plus, Sorvall Instruments, Dupont, USA), High-performance liquid chromatography (LCD Analytical, Inc., MA, USA), Hot air oven (Memmert, USA), Hot plate stirrer (Janke & Kunkel Gmbtt & Co.KG, Staufen, Germany), Moisture Analyzer (Sartorious MA 30, Germany), pH meter (Orion EA940, USA), Sieve tray (20 mesh, laboratory test sieve,

Endocotls Co., Ltd, England), Spectrophotometer (Novaspec II, Pharmacia, LKB Biochrom, England), Sonicator (Langford Electronics Ltd., Kings Norton, Birmingham, England), Suction Set (Model A-3S Tokyo rikakikai Co., Ltd, Japan), Water bath (B-480, BUCHI Labortechnik AG, Switzerland and Julabo P, Seelbach, Germany), Water bath shaker (Grant, Cambridge, England).

5. Determination of optimum condition of high performance liquid chromatography (HPLC) for 5′- nucleotide analysis

5.1 Gradient system

5.1.1 Instrumentation; HPLC consisting of a Model Consta Metric 3500 pump, a spectro Monitor 3100 detector operating at 254 nm and a Model CI-4100 recorder (LCD analytical, Inc., MA, USA) is shown in Figure 10.

5.1.2 Analytical column; column was a μBondapak C_{18} 125°A 10 μm stainless-steel column (30 cm* 3.9 mm I.D.). It was purchased from the Waters Co., Ltd, Milford, Massachusetts, USA. The column packing was silica-based 10 μm irregular beads bonded with 10 % load of C_{18} material and protected by Water guard column made of the same packing material. The column was stored in methanol (HPLC grade) when it was unused.

5.1.3 Chromatographic condition; chromatographic system was gradient system as described by Jian Zhao and Graham H.F [80]. Elution comprised of two eluents. Eluent A was a solution of 0.05 M KH_2PO_4 adjust to pH 5.45 ± 0.02 with 0.05 M K_2HPO_4. Eluent B was 100%methanol (HPLC grade). Prior to use, all eluents were filtered through a membrane filter, pore size 0.45 μm and degassed in a sonicator for 15 min.

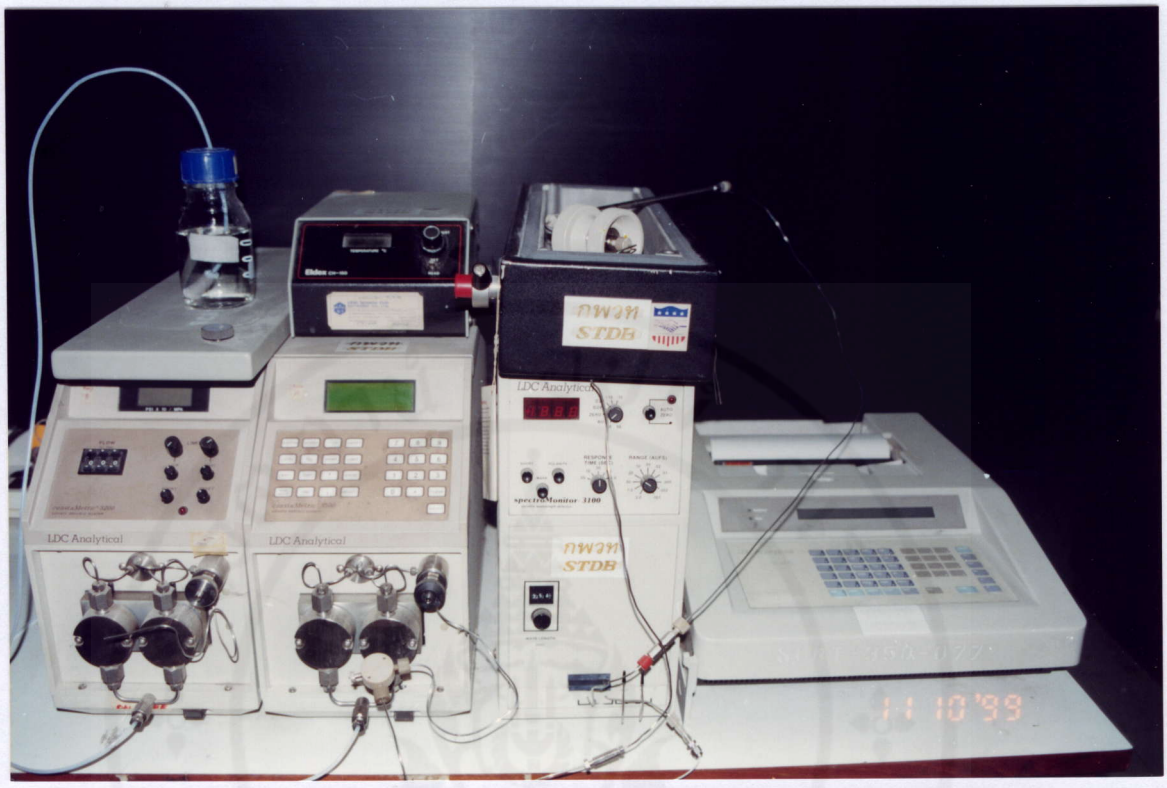


Figure 10 High-performance liquid chromatrography.

The concentration of individual standard 5′-nucleotides were prepared at 100 μg/mL. Prior to use, they were also filtered through a membrane filter, pore size 0.25 μm. Before injection of a sample, the column was equilibrated with eluent A for 10 min to stabilise the baseline. The injection volume of each standard was 60 μL. After injection, elution was started with 100% eluent A and 0% eluent B. The ratio of eluent B in the system was increased linearly from 0 to 10 % over 10 min, and the elution was maintained at this condition (90% A, 10% B) until the run was completed. The system was then returned to its starting condition (100% A) for the next run. One analysis took 15 min with the flow-rate of eluent being maintained at 1.5 mL/min throughout the run. Elution was performed at ambient temperature (approimately 25°C).

5.2 Determination of optimum increasing rate of methanol, eluent B, in gradient system for nucleotide separation

The optimum increasing rate of methanol, elution B, in gradient system was studied by linearly increasing of methanol from 0 to 10 % over various time intervals of 5 – 20 min during elution.

5.2.1 Eluent A, 0.05 M K_2HPO_4, pH 5.45 ± 0.02

A) Methanol increasing linearly from 0 to 10 % over 20 min, before injection of a sample the column was equilibrated with eluent A for 10 min to stabilize the baseline. After injection, the elution was started with 100 % eluent A and 0 % eluent B. The ratio of methanol, eluent B, was increased linearly from 0 to 10 % over 20 min (0.5 % per minute) and the elution was maintained at this condition (90% A, 10% B) until the run was completed. The flow rate of eluent being maintained at 1.5 mL/min and performed at ambient temperature.

B) Methanol increasing linearly from 0 to 10 % in 15 min, the procedure was the same as that described above except the methanol ratio, eluent B, was increased linearly from 0 to 10 % over 15 min (0.75% per minute).

C) The methanol ratio was increased linearly from 0 to 10 % over 10 minute (1% per minute).

D) The methanol ratio was increased linearly from 0 to 10 % over 5 minute (2% per minute).

5.2.2 Eluent A, 0.05 M KH_2PO_4, pH 4.45 ± 0.02

The pH of eluent A, 0.05 M KH_2PO_4, was adjusted from 5.45 ± 0.02 to 4.45 ± 0.02 with 0.05 M K_2HPO_4. The eluent B was the methanol with various

methanol increasing ratio the same as those described in A to D of the experiments 5.2.1

5.3 Comparison of 5′-nucleotide analysis obtained from gradient and isocratic system

The isocratic system was performed with one eluent. The eluent was a solution of 0.05 M KH_2PO_4 adjusted to pH 5.45 ± 0.02 with 0.05 M K_2HPO_4. Prior to use, the eluent was filtered through a membrane filter, pore size 0.45 μm and degassed by sonicator for 15 min. The injection volume was 60 μL. The flow rate of eluent being maintained at 1.5 mL/min and performed at ambient temperature.

5.4 Determination of optimum pH of mobile phase in isocratic system

The pH of mobile phase, 0.05 M KH_2PO_4, was adjusted to 4.50, 4.75, 5.00, 5.25, 5.50, 6.00 and 6.50. The flow rate of mobile phase was 1.5 mL/min and performed at ambient temperature. The sample was the standard mixture of 100 μ g/mL of 5′-GMP and 5′-IMP.

5.5 Standard curve for 5′-GMP analysis

The standard curve of 5′-GMP was obtained under the isocratic system with mobile phase of 0.05 M KH_2PO_4 pH 4.75 ± 0.02, at flow rate of 1.5 mL/min. The concentration of 5′-GMP were 25, 50, 100, 150 and 200 μg/mL. They were filled through a membrane filter (pore size 0.25 μm) before injection. The injection volume was 60 μL.

5.6 Spiking standard

To confirm the 5′-GMP identification in chromatogram, the spiking standard was used. After the sample was analyzed for 5′-GMP by HPLC, it was spiked by 5′-GMP standard and injected again into the system. The chromatograms of sample and spiked sample were compared.

6. Comparison of 5′- phosphodiesterase activity of dried malt rootlets, fresh malt rootlets and fresh rice rootlets

6.1 Cultivation of barley and rice grains in laboratory

Barley and rice grains were immersed in 10 % corlox solution for 5 min and washed 3 times with water. The cleaned barley and rice grains were then steeped into water having temperature of 10-15°C for 3 days. The water was changed two to four times during steeping period to prevent microorganism contamination [81]. After 3 days, the grains were then thinly spread on the sieves with the grains layer height of around 1 cm. These grains were covered with the moisted thick cloths and laid on the trays containing a little of water. They were sprayed every day with a solution of 0.05% (w/v) chroloform to prevent microorganism contamination. The temperature of grains cultivation was 25°C.

6.2 Crude enzyme extraction

Dried malt rootlets normally were contaminated with small pieces of broken malt grain, fine sand and dust. Before extraction, they were separated from those contaminated particles by seive (20 mesh).

One hundred and twenty grams (dry weight basis) of fresh malt rootlets, fresh rice rootlets and dried malt rootlets were homogenized with 1 L of distilled water (pH 6) at temperature of 4°C in a blender (Moulinex model X13) for 3 min. The homogenates were filtered through several layers of cloth. The turbid filtrate was centrifuged at 12,000 x g for 40 min at 4°C [6].The supernatant (malt rootlet extract) were kept at 4°C until further analysis.

6.3 Assay of 5′-phosphodiesterase activity [6]

The activity of 5′- phosphodiesterase was assayed by measurement of the rate of hydrolysis of bis (p-nitrophenyl) phosphate (Sigma,USA) [6]. The incubation mixture contained 2 μmole bis (p-nitrophenyl) phosphate, 15 μmole $MgCl_2$, 102 μmole Tris - HCl buffer, pH 8.9, and 50 μL crude enzyme solution in a total volume of 1 mL. The incubation was carried out at 37°C for 10 min. Then 2.5 mL of 0.1 M NaOH containing 20 mM EDTA was added to terminate the reaction. The absorbance at 400 nm of the p-nitrophenol fromed was determined. One unit of the enzyme was defined as the amount that catalyzed the formation of 1 μmole of p-nitrophenol per minute under the assay conditions.

6.4 Determination of protein content of crude enzyme

Protein concentration was measured by method described by Lowry [82] (see, appendix A). Bovine serum albumin was used as a standard. The concentration of BSA were 0.05, 0.1, 0.15, 0.25 and 0.35 mg/mL. The optical density was determined at 750 nm.

7. Concentration of dried malt rootlet extract by ammonium sulfate

7.1 Ammonium sulfate precipitation

The precipitation of dried malt rootlet extract by ammonium sulfate was carried out at 80% saturation and 40 - 80% saturation. Ammonium sulfate (523 g/L for 80% saturation, 229 g/L for 40% saturation and 262 g/L for 40-80% saturation, the amount of ammonium sulfate were calculated from Table 4.10, see appendix B) was slowly added into dried malt rootlet extract at 40% and 80% saturation whilst stirring at 4°C. Leave each aliquot further stirred overnight at 4°C. Centrifuge each aliquot at 3,000 x g for 40 min at 4°C [80]. After centrifugation, the pellet of 80% saturation was resuspended in small volumes of sterile distilled water, approximately twice the volume of the pellet. For 40% saturation, the supernatant was again slowly added by ammonium sulfate (262 g/L) and leave further stirred overnight at 4°C. Centrifuge the solution at 3,000 x g for 20 min at 4°C. The pellets were resuspended in small volumes of sterile distilled water. Salts were removed from both resuspensions by dialysis.

7.2 Dialysis

Dialysis tube (Cell-Sep T3 MWCO: 12,000–14,000) was cut into suitable lengths having a volume required. The tube was submerged in distilled water for 15 min, transferred to a solution of 0.05% EDTA at 80°C for 30 min with slow stirring. The tube was then moved into stirred distilled water at 80°C for 30 min, cooled and placed in a solution of 0.05% sodium azide to prevent microbial growth.

The dialysis tube was rinsed inside and outside with distilled water and sealed one end of the tube with dialysis clip. The solution to be dialyzed was poured in the tube, expeled the air from the bag and sealed the top end of the tube with dialysis

clip. The bag was placed in a large volume of distilled water, 100 to 1,000 times volume of original volume, agitated gently. Leave to reach equilibrium, usually 3 hr, preferably at 4°C [80]. The concentrated malt rootlet extract were then analyzed for the protein content and enzyme activity by Lowry method and 5′- phosphodiesterase activity assay, respectively.

8. Preparation of 5′-GMP-rich yeast extracts

The preparation process was divided into two parts: yeast autolysis and enzymatic hydrolysis.

8.1 Determination of optimum yeast autolysis time for preparation of 5′-GMP-rich yeast extracts

8.1.1 Yeast autolysis : brewer's yeast slurry containing 15-20 % total solid (dry weight basis) was adjusted to 15 % total solid using sterile distilled water and adjusted to pH 5 with 2.0 N NaOH or 2.0 N HCl solution as appropriate. Three hundred mL of yeast slurry was transferred to six 1 L Erlenmeyer flasks which were then incubated at 50°C in a stroke water bath operating at 200 strokes/min. Yeast autolysis time was varied from 4 to 24 hr. Samples were taken at 4, 8, 12, 16, 20 and 24 hr of incubation, heated at 80°C for 20 min to terminated the reactions.

8.1.2 RNA extraction and cell debris separation: yeast autolysates obtained at each autolysis time were adjusted to pH 6.2-6.4, incubated at 95°C for 2 hr in a stroke water bath operating at 200 strokes/min. Autolysates were then centrifuged at 15,000 x g for 15 min at 4°C to separate cell debris. The supernatants (yeast extract solutions) were kept at 4°C until used in the further step.

8.2 Determination of optimum enzymatic hydrolysis time and concentration of enzyme

8.2.1. Enzymatic hydrolysis; yeast autolysates obtained at various autolysis time were mixed with concentrated dried malt rootlet extract at a ratio of 100 mL of yeast autolysate (5 % total solid dry weight basis) and enzyme at various concentrations (20, 60, 100, 140 unit). A sample without enzyme addition was used as a reference. The mixtures were adjusted to pH 6.2-6.4, incubated at 60°C in a stroke water bath operating at 200 strokes/min. The enzymatic hydrolysis times were 2, 4, 8, 14, 20, 26 and 44 hr.

8.2.2 Sampling and analysis; samples were taken from incubation flasks at each specified time interval, heated at 95°C for 5 min. The samples were analyzed for solids content and 5′- GMP content. Prior to injecting the samples into the HPLC system, they were filtered through a nylon filter membrane (pore size 0.25 μm) and diluted at appropriate dilution.

9. Preparation of 5′-GMP-rich yeast extracts from debittered spent brewer's yeast

9.1 Debittering of brewer's yeast cells

Brewer's yeast slurry was centrifuged at 10,000 x g for 10 min at 4°C to remove the beer liquor. The yeast paste was adjusted to 15 % solids content with distilled water and adjusted to pH 9-10 with 2% Na_2CO_3 at 20°C. The suspension was stirred for 30 min and then centrifuged at 10,000 x g for 10 min at 4°C. The yeast cell paste was washed with distilled water until the pH was neutral. It was adjusted to 15 % solids content and used as raw material for further process.

9.2 Debittered yeast autolysis and enzymatic hydrolysis

The autolysis of debittered yeast was performed with the same procedure as non-debittered yeast (8.1). Enzymatic hydrolysis of debittered brewer's yeast was also preformed as non-debittered yeast (8.2) except enzyme hydrolysis time were 0, 1, 2, 4, 8, 14, 20 and 26 hr.

10. 5′-Nucleotidase inactivation

10.1 Determination of suitable conditions for pre-treatment malt rootlet extract

To inactivate 5′- nucleotidase in malt rootlet extract, various conditions from the literatures were chosen for study. These conditions were heating malt rootlet extract at 63 °C for 1 hr, 72°C for 5 min, 72°C for 5 min along with adding inhibitor 0.005 M $Zn.Ac_2.H_2O$, 85°C for 7 and 30 min, 95 °C for 5 min, 100°C for 10 and 30 min, adding inhibitor (0.05 M $Zn.Ac_2.H_2O$). Non-pretreatment concentrated crude enzyme was used as a control. Solution of 0.1% (w/v) 5′- GMP 100 mL was mixed with 10 mL malt rootlet extract which pre-treated at various conditions as mention above. The mixture was adjusted to pH 6.2-6.4, incubated in a stroke water bath operating at 200 strokes/min at 60 °C. The enzyme hydrolysis time were 0 hr (as control) 2, 4, 8, 14 and 20 hr. At periodic intervals, samples were taken from incubation flasks and increased the temperature to 95°C for 5 min.The solution of 0.1 % (w/v) 5′- GMP, pH 6.2-6.4, was also incubated for use as a reference of the effect of incubation condition on 5′- GMP stability. The remaining of 5′- GMP of the samples was assayed.

10.2 Determination of optimum pH for 5′- phosphodiesterase activity

The pH of dried malt rootlet extract was adjusted to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Then assayed for 5′-phosphodiesterase activity.

10.3 Determination of optimum heating time and temperature for 5′-nucleotidase inactivation

For 5′- nucleotidase inactivation, the malt rootlet extract was heated at various times and temperatures. The temperatures ranged from 60 to 80°C and heating times ranged from 2 to 180 min. At 60°C; heating time were 60, 120, 160 and 180 min. At 65°C; heating times were 30, 60, 120 and 160 min. At 70°C; heating times were 7, 20, 40 and 60 min. At 75°C; heating times were 5, 10, 20 and 30 min. At 80°C; heating times were 2, 5, 10 and 15 min.

Each pre-treated extract was divided into two portions. The first portion was assayed for 5′- phosphodiesterase activity and the rest was mixed with a solution of 0.1 % (w/v) 5′- GMP at a ratio of 1:10. The mixture (20 mL in 125 mL Erlenmeyer flask) was adjusted to pH 6.2-6.4 and incubated in a stroke water bath operating at 200 strokes/min at 60°C for 8 hr. A solution of 0.1 % (w/v) 5′-GMP and non-pretreated extract pH 6.2-6.4 was also incubated for use as a reference. All samples were analyzed for remaining 5′- GMP.

10.4 Using heat pre-treated extract in preparation of yeast extracts

The optimum condition of heat pre-treated extract was used in preparation process of yeast extract. The procedure was performed with the same procedure as experiment 9.1 and 9.2 except the autolysis time was carried out at 8 hr.

11. Effect of phosphodiesterase concentration on 5′-GMP level

Debittered brewer's yeast slurry was adjusted to 15 % solids content, pH 5, incubated at 50°C for 8 hr. The autolysate obtained was adjusted to pH 6.2-6.4, incubated at 95°C for 2 hr and then centrifuged at 15,000 x g for 15 min at 4°C.

The yeast extract (100 mL of 5 % solids) was mixed with heat pre-treated phosphodiesterase at various concentration i.e., 100, 160, 230 and 300 unit. A sample without enzyme addition was used as a control. The mixture were adjusted to pH 6.2-6.4 and incubated at 60°C for 8 hr in a stroke water bath operating at 200 strokes/min. The enzymatic reaction was terminated by heating the samples at 95°C for 5 min. The 5′- GMP content of each sample was then analyzed.

12. Determination of RNA content

12.1 RNA analysis

RNA was determined according to a procedure adapted from Herbert *et al.*, (1971) [74]. Twenty mg (dry weight basis) of sample was added with 10 mL of 0.5 M $HClO_4$, well mixed and incubated in water bath at 90°C for 20 min. After cooling, the sample was filtered through a filter paper No. 42 (Whatman, USA). The filtrate was analyzed for the RNA content using spectrophotometer at absorbance of 260 nm.

12.2 Determination of RNA content in sample of 5′-GMP-rich yeast extracts

12.2.1 Preparation; yeast paste of both before and after debittering were analyzed the RNA content by RNA analysis.

12.2.2 Autolysis and RNA extraction; three hundred mL of debittered yeast paste containing 15 % solids, pH 5, was transferred to 1 L Erlenmeyer flask and incubated in a stroke water bath operating at 200 strokes/min at 50°C for 8 hr. The autolysate obtained was divided into two portions; the first portion (100 mL) was sterilized at 80°C for 20 min and then centrifuged at 15,000 x g for 15 min at 4 °C. The cell debris and yeast extract were analyzed for the RNA content. The second portion (200 mL) was adjusted to pH 6.2-6.4, transferred to 1 L Erlenmeyer flask and incubated in a stroke water bath operating at 200 strokes/min at 95°C for 2 hr. After that, centrifuged at 15,000 x g for 15 min at 4°C. The yeast extract and cell debris were analyzed for the RNA content.

12.2.3 Enzyme hydrolysis; the yeast extract (5% total solid dry weight basis) 100 mL was mixed with 100 unit of heat pre-treated extract (70°C for 7 min), adjusted to pH 6.2-6.4 and incubated in a stroke water bath operating at 200 strokes/min at 60°C. Samples were taken at 0, 2, 4, 8, 14 and 20 hr of incubation. All samples were analyzed RNA and 5′-GMP content.

12.3 RNA content in commercial yeast extracts

The commercial yeast extracts; Maxarome standard powder, Quest, Gistex AGGL and Maxavor Rye-B Powder were also analyzed for their RNA content.

13. Study of 5′- GMP produced from pure substrate RNA

RNA (from baker's yeast) were prepared as starting substrate solutions having concentration of 0.5 %, 1.0 % and 1.5 % (w/v). Concentrated malt rootlet extract was heated at 70°C for 7 min and then assayed for 5′- phosphodiesterase

activity. One hundred units of 5′- phosphodiesterase activity were added into the 0.5 %, 1.0 % and 1.5 % (w/v) RNA solution (100 mL) in 250 Erlenmeyer flasks, adjusted to pH 6.2-6.4, incubated in a stroke water bath operating at 200 strokes/min at 60 °C. Samples were taken at 0 hr (as control), 2, 4, 8, 14 and 20 hr of incubation, heated at 95°C for 5 min to terminate the reaction. The 5′- GMP was then analyzed.

14. Statistic analysis

The data was calculated as mean and standard deviation (SD). Treatment means were compared by one way analysis of various (ANOVA) with a significant level of 95 %. Also, the mean values were computed and compared by using Dunean New Multiple Range test. Statistic analytical program was statistical packages for the social science (SPSS) version 7.5 for windows.

CHAPTER IV

RESULTS

1. Determination of optimum condition of high performance liquid chromatography (HPLC) for 5′-nucleotide analysis

1.1 Gradient system

HPLC analysis of an individual standard nucleotide was performed by gradient system with eluent A was 0.05 M K_2HPO_4, pH 5.45 ± 0.02 and eluent B was 100% methanol. Their chromatograms are shown in Figure 11. From these chromatograms, the sequence of elutions were 5′-UMP, 5′-IMP, 5′-GMP, 5′-AMP, 3′-UMP and 2′-GMP at retention time 2.85, 4.13, 4.16, 6.89, 7.73 and 10.14 min, respectively.

Figure 12 is a chromatogram of a standard mixture of nucleotides. The individual peak of a standard mixture of nucleotides was identified by matching their respective retention time with those of standard compounds injected individually as shown in Figure 11. The retention times of 5′-IMP and 5′-GMP were very close, so they were simultaneously eluted at a retention time of 4.11 min.

According to the chromatogram, this chromatographic condition seem to be not appropriate for the nucleotide analysis because the 5′-IMP and 5′-GMP were not separated and eluted as a one peak in this condition. Owing to this result, the flow rate of eluent was reduced with expect to retain the elution of 5′-GMP and 5′-IMP and separate them from each other. The flow rate was declined from1.5 to 1.0 mL/min. The chromatogram is shown in Figure 13.

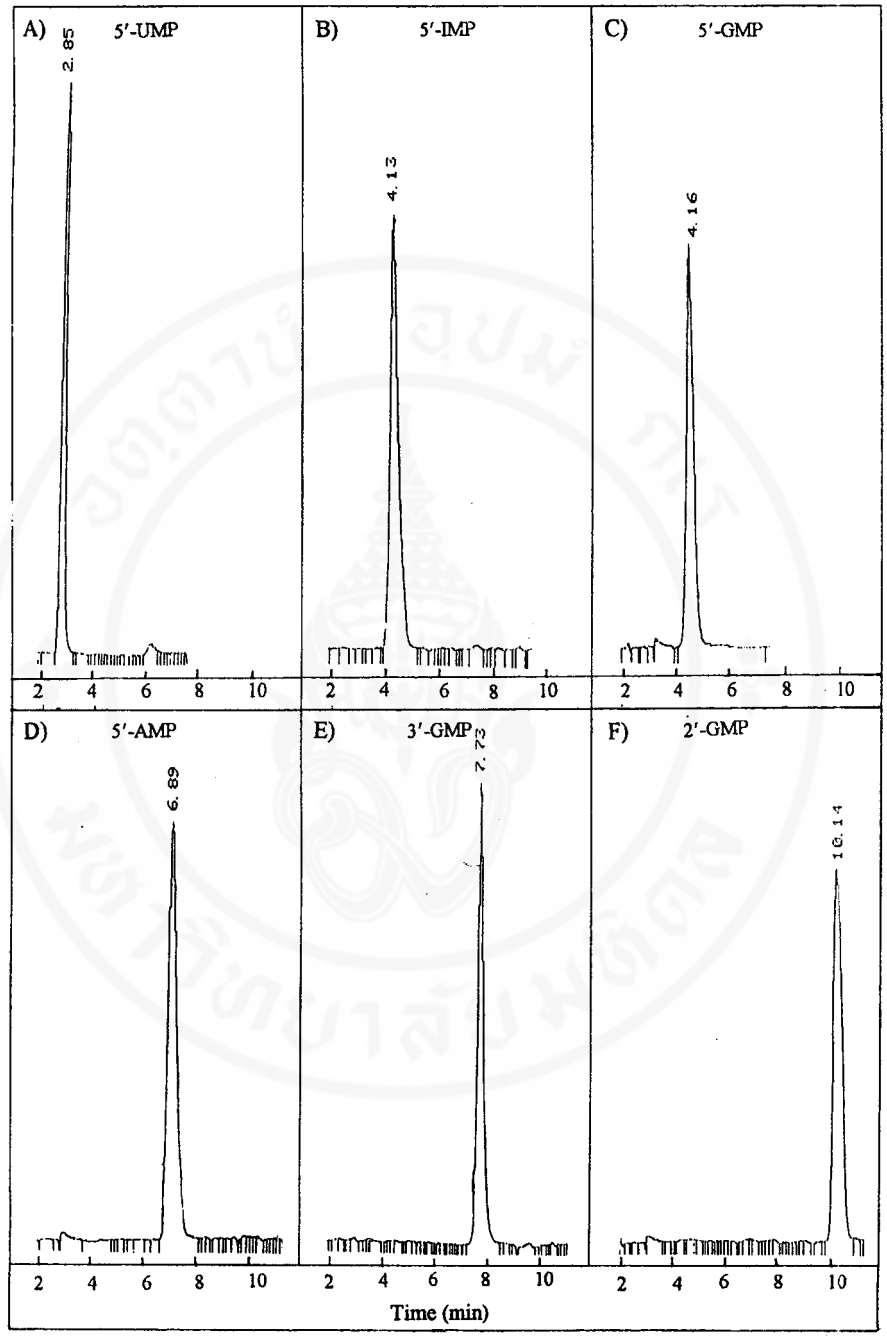


Figure 11 HPLC chromatograms of an individual standard nucleotide obtained from gradient system. Column, Water μ Bondapak C_{18}; temperature, ambient. Eluent A, 0.05 M KH_2PO_4, pH 5.45±0.02; eluent B, 100% methanol. Elution program: ratio of eluent B was increased linearly from 0 to 10% over 10 min; flow rate, 1.5 mL/min; detection wavelength, 254 nm; injection volume, 60 μL [81].

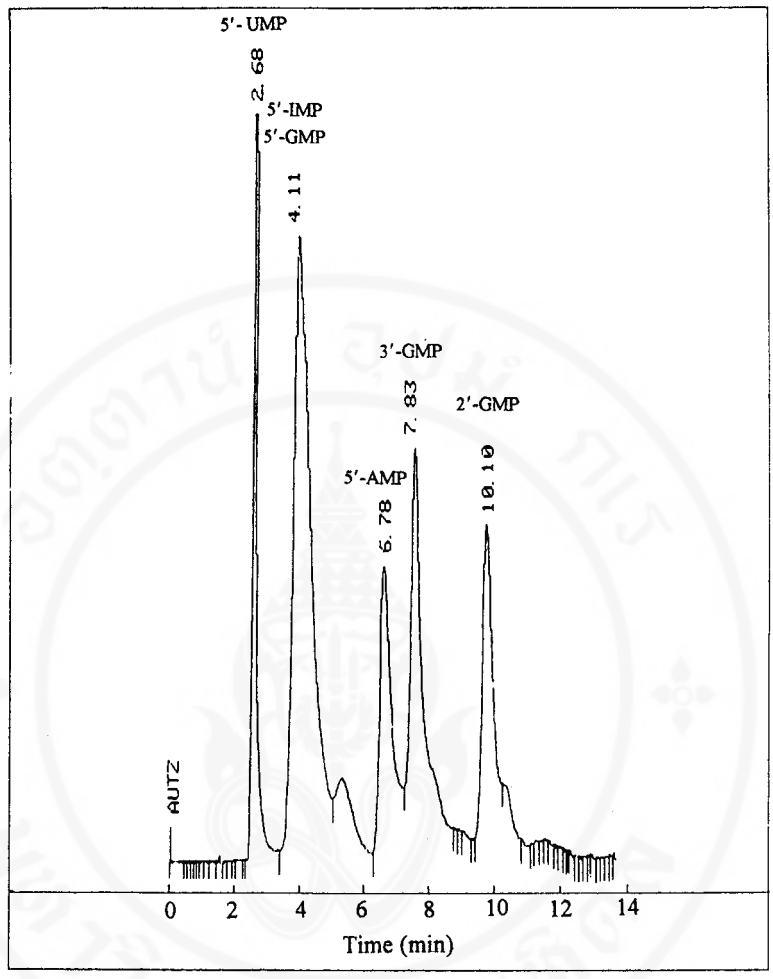


Figure 12 HPLC chromatogram of a standard mixture of nucleotides obtained from gradient system. Column, Water μ Bondapak C_{18}; temperature, ambient. Eluent A, 0.05 M KH_2PO_4, pH 5.45±0.02; eluent B, 100% methanol. Elution program: ratio of eluent B was increased linearly from 0 to 10% over 10 min and held at this condition until the run was finish; flow rate, 1.5 mL/min; detection wavelength, 254 nm; injection volume, 60 μL.

The Figure shows that decreasing the flow rate of eluent from 1.5 to 1.0 mL/min resulted in prolonging the elusion times such as the retention time of 5′-UMP was shifted from 2.68 to 3.85 min. The elution sequence of a standard mixture of

nucleotides at flow rate 1.5 mL/min was the same sequence as those of flow rate 1.0 mL/min. Nevertheless, it can be slightly improved the resolution of 5′-GMP and 5′-IMP.

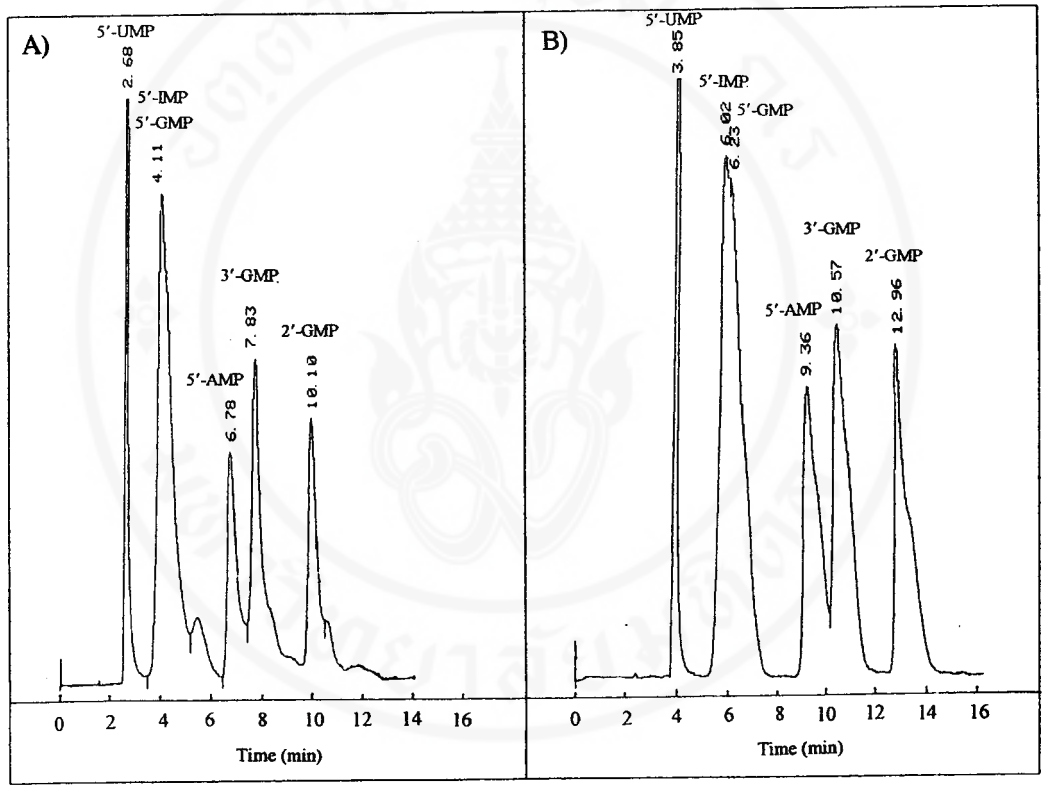


Figure 13 Comparison of HPLC chromatograms of a standard mixture of nucleotides from gradient system at flow rate of eluent 1.5 mL/min (A) and 1.0 mL/min (B). Column, μ Bondapak C_{18}; temperature, ambient. Eluents: eluent A, 0.05 M KH_2PO_4, pH 5.45±0.02; eluent B, 100% methanol. Elution program: ratio of eluent B was increased linearly from 0 to 10% over 10 min and held at this condition until the run was finish; detection wavelength, 254 nm; injection volume, 60 μL.

1.2 Determination of optimum increasing rate of methanol, eluent B, in gradient system for nucleotides separation

The methanol ratio, eluent B, in the elution of gradient system considered as one of the important parameters for improving the resolution of standard nucleotides, especially 5′-GMP and 5′-IMP. The methanol ratio was linearly increased from 0 to 10 % in 20, 15, 10 and 5 min, respectively (methanol increasing in eluent A was 0.5%, 0.75%, 1% and 2% per min). The HPLC chromatograms are shown in Figure 14.

The chromatograms show that increasing the methanol ratio trend to decrease the elution time of standard nucleotides. For instance, the retention time of 2′-GMP was decreased from 12.54 min at methanol increasing rate of 0.5% per min to 10.93, 9.45 and 9.21 min at methanol increasing rate of 0.75, 1 and 2% per min, respectively. The elution sequence of nucleotides of all methanol increasing rates were unchanged and found that at higher of methanol increasing ratio the peak base was shaper.

The different elution time between the first peak (5′-UMP) and the last peak (2′-GMP) of a standard mixture of nucleotides was also the one of parameter for studying the effect of methanol increasing ratio on nucleotide analysis efficiency. The different elution times were 9.76, 8.10, 6.53 and 6.47 min at methanol increasing rates of 0.5, 0.75, 1.0 and 2.0% per min, respectively. The relationship between these two parameters is shown in Figure 15.

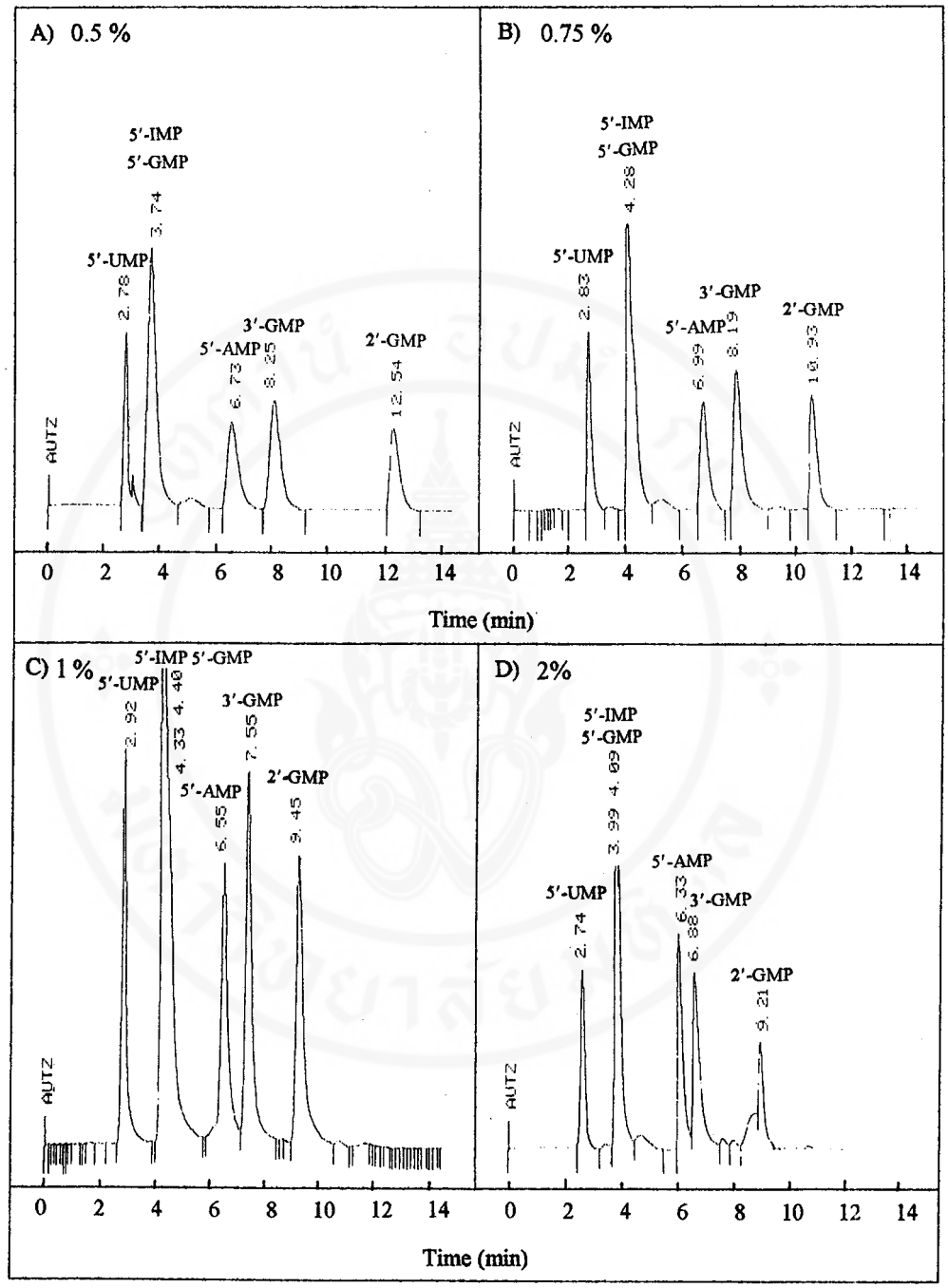


Figure 14 HPLC chromatograms of standard mixture of nucleotides obtained from gradient system. Column, Water μ Bondapak C_{18}; temperature, ambient. Eluent A, 0.05 M KH_2PO_4, pH 5.45±0.02; eluent B, 100% methanol. Elution program: ratio of eluent B was increased linearly from 0 to 10% over 20 min (A), 15 min (B), 10 min (C) and 5 min (D); flow rate, 1.5 mL/min; injection volume, 60 μL.

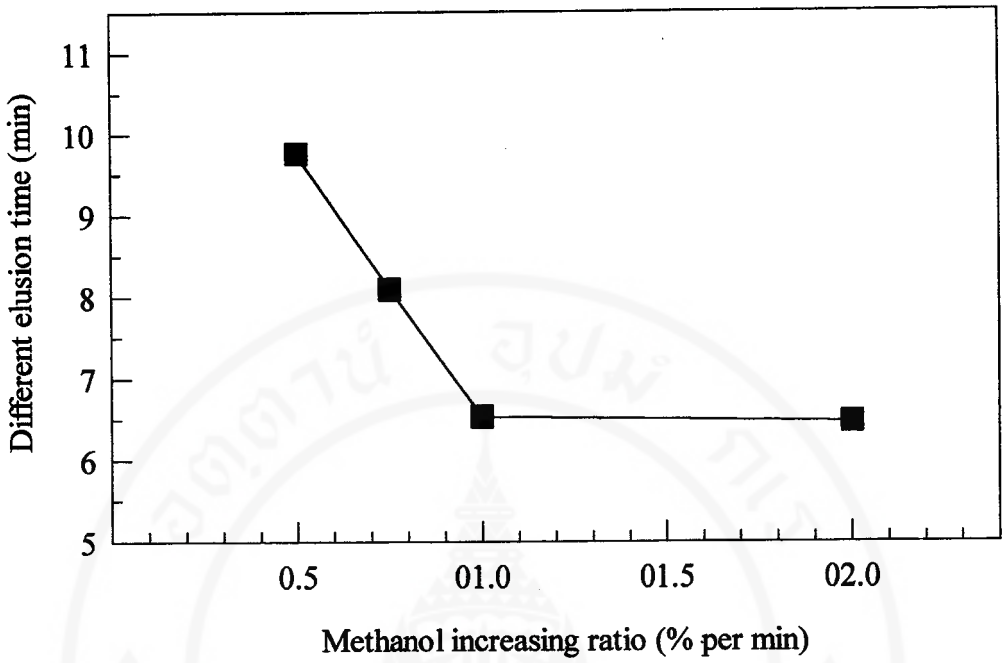


Figure 15 Relationship of methanol increasing ratio (% per min) and different elution time between the first and the last elution of gradient system.

From Figure 15, the different elution times between the first and the last peak were sharply declined with increasing of methanol ratio from 0.5 to 1.0% per min and then leveled off. It was implied that the methanol increasing ratio over than 1% per min was not effect the standard nucleotide elution efficiency.

Although the results were engendered the comprehension about the effect of methanol increasing ratio on standard nucleotides elution efficiency, however the objective of this study was unsuccessful. They were failed to improve the resolution of 5′-GMP and 5′-IMP, although the methanol increasing at 1% and 2% per min seem to be better than those of 0.5% and 0.75% per min because the peak of 5′-GMP and 5′-IMP were occasionally separately detectable as two peaks by detector. Nevertheless, this condition was not suitable for choosing as condition for nucleotide analysis.

1.3 Decreasing of pH of eluent A, 0.05 M KH_2PO_4, at various methanol increasing ratio, eluent B, in the elution of gradient system

The pH of eluent A, 0.05 M KH_2PO_4, in gradient system was decreased from 5.45±0.02 to 4.50±0.02. The eluent B was the methanol at various methanol increasing ratio the same as experiment 5.2.1. The results of this experiment are shown in Figure 16. Comparison of chromatograms from Figure 14 and Figure 16 show that the resolution of chromatograms from Figure 16 (eluent A pH 4.45) was poorer than those from Figure 14 (eluent A pH 5.45) because not only they were not improved the resolution of 5′-GMP and 5′-IMP but the peak of 5′-AMP and 3′-GMP were shifted closer and took a longer elution time as well. Therefore, decreasing the pH of eluent A from 5.45 to 4.45 were not appropriate for nucleotide analysis.

1.4 Comparison of nucleotide analysis between gradient system and isocratic system

The isocratic system was studied for comparation with the gradient system for nucleotide analysis. The isocratic system was performed with one eluent i.e., 0.05 M KH_2PO_4, pH 5.45 at flow rate of 1.5 mL/min at ambient temperature. The gradient system was carried out as precedure described in 5.1. The sample was the standard nucleotide mixture. The HPLC chromatograms of isocratic and gradient systems are shown in Figure 17.

Comparison of nucleotide analysis efficiency between isocratic system and gradient system shows that the separation efficiency of both systems were not quite different. The elusion sequence was the same but the retention time of each nucleotide was longer in the isocratic system than those of the gradient system.

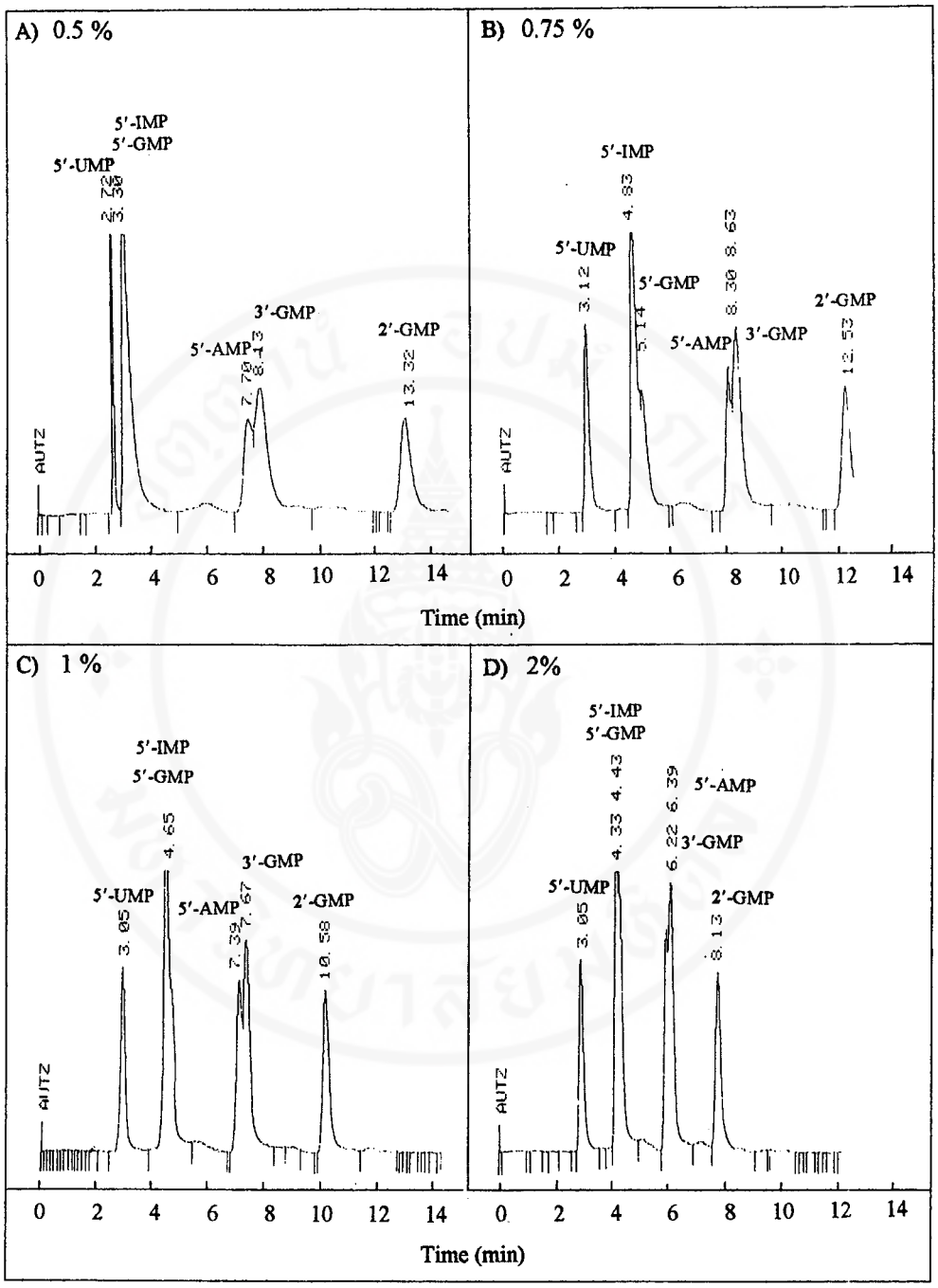


Figure 16 HPLC chromatograms of standard mixture of nucleotides obtained from gradient system. Column, Water μ Bondapak C_{18}; temperature, ambient. Eluent A, 0.05 M KH_2PO_4, pH 4.45±0.02; eluent B, 100% methanol. Elution program: ratio of eluent B was increased linearly from 0 to 10% over 20 min (A), 15 min (B), 10 min (C) and 5 min (D); flow rate, 1.5 mL/min; injection volume, 60 μL.

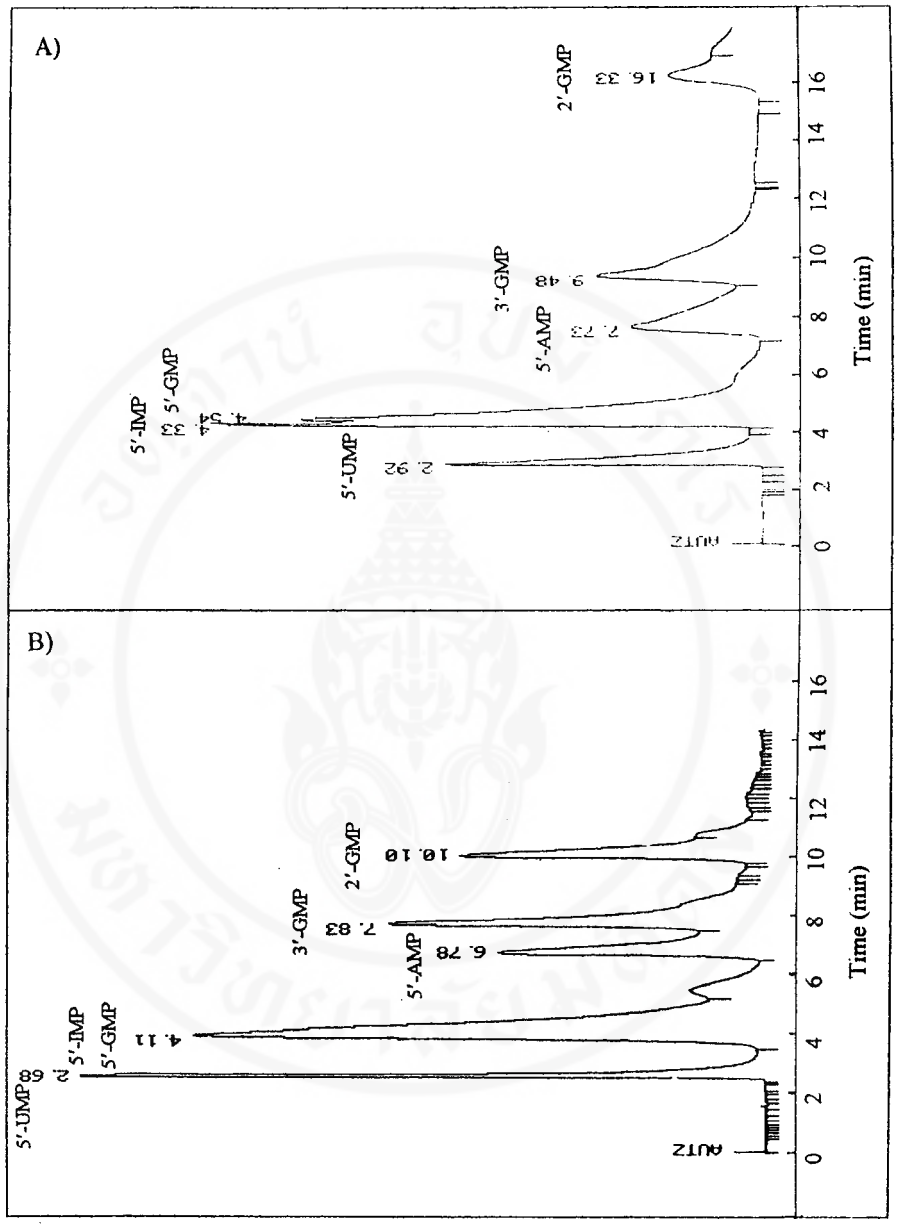


Figure 17 HPLC chromatograms of standard mixture of nucleotides. Column, Water μ Bondapak C_{18}; temperature, ambient. A: isocratic system; eluent was 0.05 M KH_2PO_4, pH 5.45±0.02. B: gradient system; eluent A, 0.05 M KH_2PO_4, pH 5.45±0.02; eluent B, 100% methanol. Elution program: ratio of eluent was increased linearly from 0% to 10% over 10 min; flow rate, 1.5 mL/min; detection wavelength, 254 nm; injection volume, 60 μL.

In consideration of the 5′-GMP and 5′-IMP resolution of both systems, the isocratic system was slightly better than the gradient system because the 5′-GMP and 5′-IMP peaks were detected as an individual peak while those of the gradient system 5′-GMP and 5′-IMP were eluted as one peak.

On account of these results, although the nucleotide resolution of the isocratic system was not quite good, it was reasonable to choose this system for further study due to its simplier and easier to operate.

1.5 Determination of optimum pH of mobile phase of the isocratic system

To improve the resolution of 5′-IMP and 5′-GMP of the isocratic system, the effect of pH of mobile phase to the resolution was studied. The pH of mobile phase, 0.05 M KH_2PO_4, was adjusted to 4.50, 5.00, 5.50, 6.00 and 6.50 respectively. The sample was the standard mixture of 5′-IMP and 5′-GMP. The HPLC chromatograms are displayed in Figure 18. The different retention time between 5′-IMP and 5′-GMP were 0.35, 0.36, 0.31, 0.21 and 0 min at pH 4.50, 5.00, 5.50, 6.00 and 6.50, respectively. The results show that increasing the pH of mobile phase cause decreasing the resolution of 5′-IMP and 5′-GMP. At mobile phase ranging from pH 4.50 to 5.50 the resolution efficiency was not significantly different. The resolution was poorer at pH more than 6.00.

From these results, it can be concluded that the optimum pH of the mobile phase of the isocratic system ranged from 4.50 and 5.50. Therefore, the mobile phase pH 4.75±0.02 was chosen for further study.

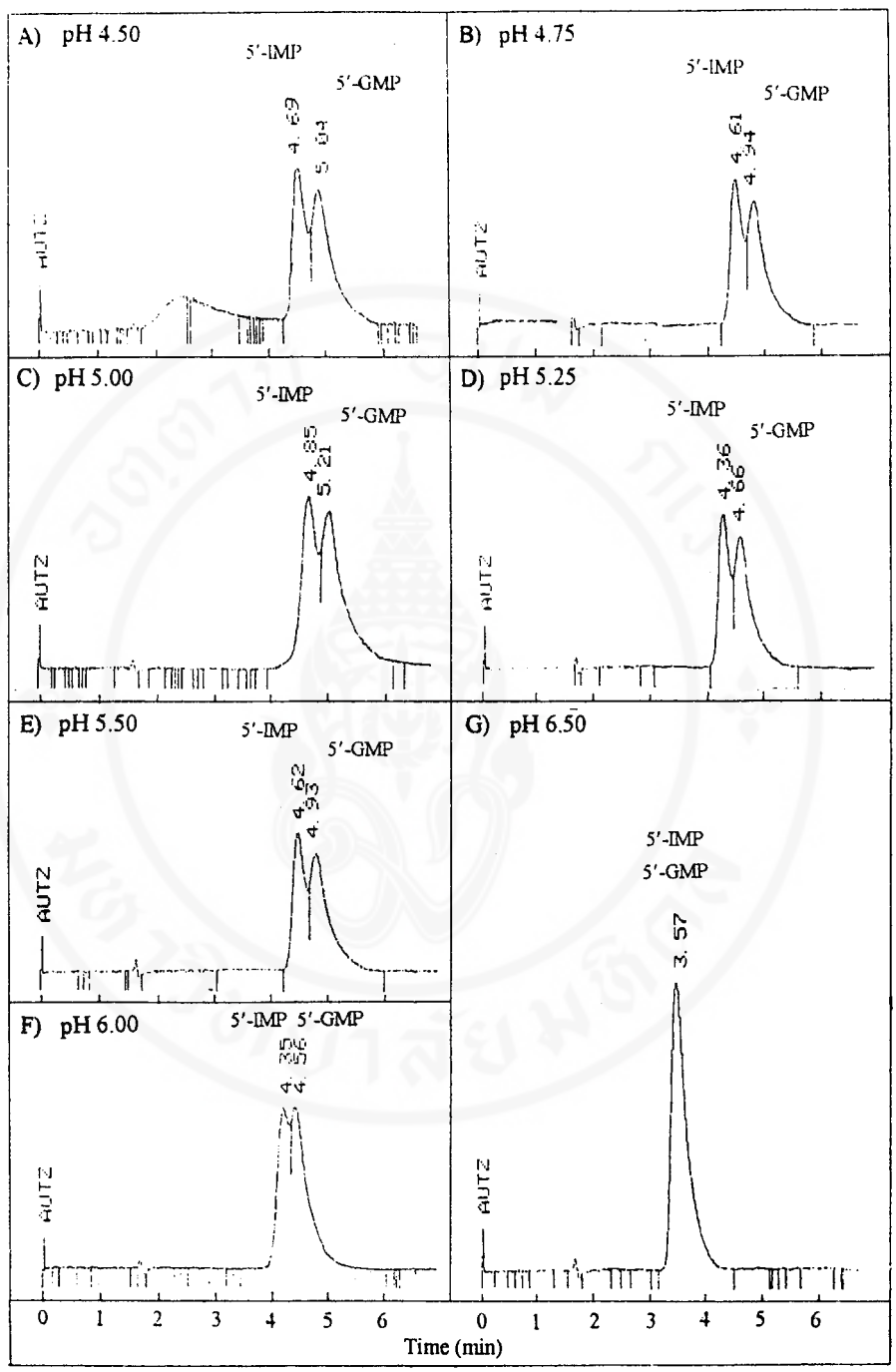


Figure 18 HPLC chromatograms of standard mixture of 5′-GMP and 5′-IMP obtained from isocratic system. Column, Water μ Bondapak C_{18}; temperature, ambient. Eluent was 0.05 M KH_2PO_4, pH 4.50±0.02 (A), 4.75±0.02 (B), 5.00±0.02 (C), 5.25±0.02 (D), 5.50±0.02 (E), 6.00±0.02 (F) and 6.50±0.02 (G); flow rate, 1.5 mL/min; detection wavelength, 254 nm; injection volume, 60 μL.

1.6 Preparation of 5′-GMP standard curve

Standard curve of 5′-GMP was prepared using the isocratic system with the mobile phase, 0.05 M KH_2PO_4 pH 4.75±0.02. The flow rate of mobile phase was 1.5 mL/min at wavelength 245 nm. The elution was operated at ambient temperature. The standard curve of 5′-GMP was obtained by plotting the peak areas against the concentration of the 5′-GMP.

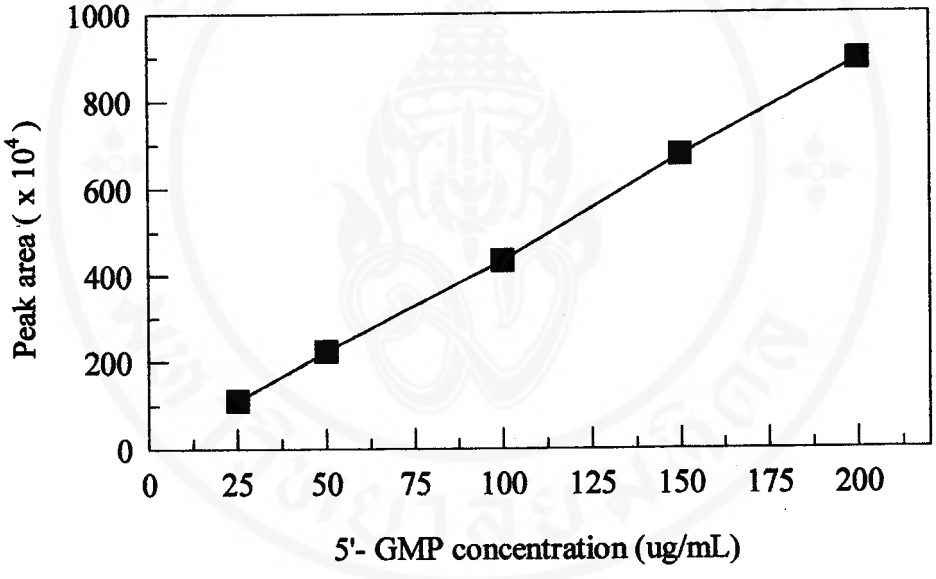


Figure 19 Standard curve of 5′-GMP

1.7 Spiking standard

The spiking standard method was used for 5′-GMP analysis of yeast extract. Figure 20 was the sample of spiking standard method of yeast extract. Picture A is a chromatogram of yeast extract before adding 5′-GMP standard, the expected peak of 5′-GMP was the peak at retention time of 3.79 min (compared with individual standard 5′-GMP) having a peak area of 1,651,352.

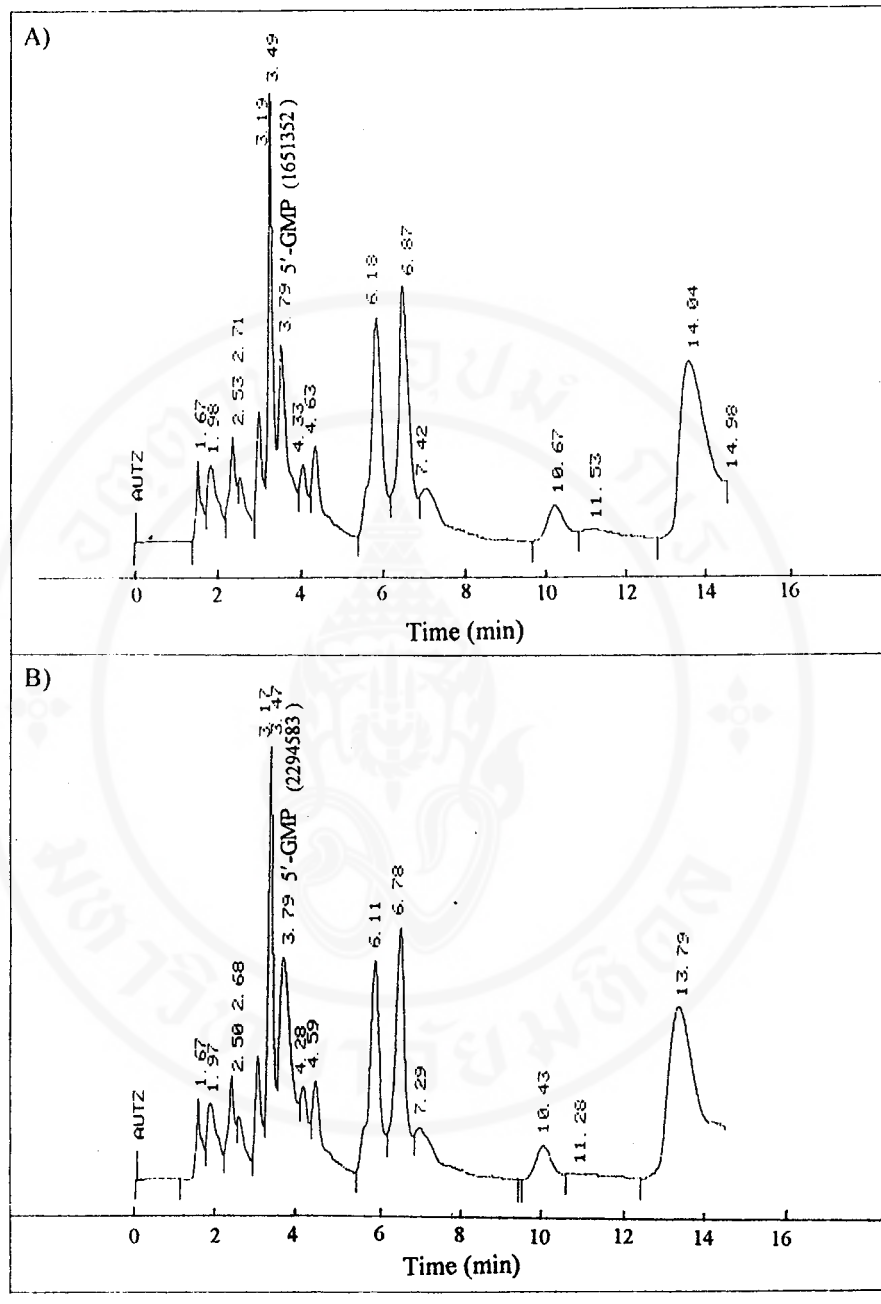


Figure 20 HPLC Chromatograms of spiking standard method obtained from gradient system. (A) Chromatogram before adding 5′-GMP standard, (B)Chromatogram after adding 5′-GMP standard. Column, Water μ Bondapak C_{18}; temperature, ambient. Eluent A, 0.05 M KH_2PO_4, pH 5.45±0.02; eluent B, 100% methanol. Elution program: ratio of eluent B was increased linearly from 0 to 10% over 10 min; flow rate, 1.5 mL/min; injection volume, 60 μL.

To identify the peak of 5′-GMP, the sample was spiked with standard 5′-GMP and injected into the HPLC system. Picture B is a chromatogram of yeast extract after adding 5′-GMP standard The peak area at retention time of 3.79 min was increased to 2,294,583.

2. Comparison of 5′- phosphodiesterase activity of dried malt rootlets, fresh malt rootlets and fresh rice rootlets.

2.1 Determination of suitable cultivation time of barley and rough rice

The cultivation of barley and rough rice was carried out in the laboratory. At the first day of cultivation, the length of barley rootlets was approximately 1-2 mm and around 1 cm at the second day. They were too small to harvest for enzyme extraction. Until the third day, taproots expanded with the length approximately 3 cm and full of branch roots. They were harvested and extracted for a crude enzyme. The 5′- phosphodiesterase activity and protein content of rootlet extract were assayed. At the fourth and fifth day, barley rootlets were also extracted and assayed. The results are shown in Table 1. Noticeably, at the fourth day of cultivation the leaves started to germinate and the taproots were slowly expanded.

In case of rough rice, the rootlets germination time was longer than barley. The grains started to germinate at the fifth day. A rice taproot was thinner and a branch root was shorter than barley rootlet. At the seventh, eighth and ninth day, the rice rootlets were harvested and extracted for a crude enzyme. They were assayed for 5′- phosphodiesterase activity and protein content. The results are displayed in Table 2.

Table 1 Solids content of fresh malt rootlets; 5′-phosphodiesterase activity, protein content and specific activity of crude extract from fresh malt rootlets at various germination times

Germination time (days)	Solids content of rootlets (%)	Activity (unit/mL)	Protein (mg/mL)	Specific* activity (unit/mg)
3	7.43 ± 0.19	2.13 ± 0.08	21.67 ± 2.32	0.098[a**]
4	7.53 ± 0.06	1.41 ± 0.25	14.80 ± 0.54	0.095[a]
5	8.72 ± 0.14	2.02 ± 0.22	22.15 ± 0.35	0.091[a]

* Mean ± standard deviation (SD) with n = 6

** Means values within columns with similar superscripts are not significantly different (P > 0.05)

Table 2 Solids content of fresh rice rootlets; 5′-phosphodiesterase activity, protein content and specific activity of crude extract from fresh rice rootlets at various germination times

Germination time (days)	Solids content of rootlets (%)	Activity (unit/mL)	Protein (mg/mL)	Specific* activity (unit/mg)
7	11.18 ± 0.16	0.46 ± 0.04	11.02 ± 0.66	0.042[a**]
8	16.91 ± 0.06	0.49 ± 0.02	12.24 ± 1.26	0.040[a]
9	21.24 ± 0.27	0.43 ± 0.02	10.48 ± 0.50	0.041[a]

* Mean ± standard deviation (SD) with n = 6

** Means values within columns with similar superscripts are not significantly different (P > 0.05)

For fresh malt rootlets, (Table 1) a specific activity of crude extract at the third, fourth and fifth day of cultivation was slightly decreased from 0.098 to 0.091 unit/mg with increasing the germination time. However, they were not statistically significant different ($p > 0.05$). From these results, it can be concluded that the specific enzyme activity of fresh malt rootlets was not effect by cultivation times.

For rice rootlets (Table 2), a specific activity of crude extract at the seventh, eighth and ninth day ranged from 0.040-0.042 unit/mg. They were also shown not statistically significant different ($p > 0.05$). Therefore, at the third and seventh day of cultivation were suitable time of barley and rice, respectively. This preferably due to the minimizing cost and process time.

2.2 Determination of the preferable source of enzyme

Dried malt rootlets was extracted for a crude enzyme and assayed for enzyme activity and protein content (Table 3). A comparison of specific activity of crude extract from various types of rootlets is shown in Table 4. The highest enzyme activity of 0.095 unit/mg was obtained from fresh malt rootlets followed by 0.066 unit/mg from dried malt rootlets. The lowest activity was 0.041 unit/mg obtained from fresh rice rootlets. These results were statistically significant different ($P \leq 0.05$).

According to these results, fresh malt rootlets seem to be the most preferable source of 5′-phosphodiesterase due to their highest specific activity. However, dried malt rootlets were chosen as a source of enzyme due to their lowest cost and also making an added value to a by product from malting industry.

Table 3 Solids content of dried malt rootlets; 5′-phosphodiesterase activity, protein content and specific activity of crude extract of dried malt rootlets

Solids content of rootlets (%)	Activity (unit/mL)	Protein (mg/mL)	Specific activity* (unit/mg)
92.60 ± 0.16	1.04 ± 0.02	15.77 ± 0.35	0.066

* Mean ± standard deviation (SD) with n = 6

Table 4 Specific activity of crude extract of various types of rootlets

Type of rootlets	Specific activity (unit/mg)
Fresh malt rootlets	0.095 ± 0.003^{a}
Fresh rice rootlets	0.041 ± 0.008^{b}
Dried malt rootlets	0.066 ± 0.001^{c}

$^{a, b, c}$ Means values within columns with different superscripts are significantly different ($P \leq 0.05$)

3. Concentration of crude dried malt rootlet extract by ammonium sulfate

Crude malt rootlet extract was concentrated by ammonium sulfate precipitation at 80% saturation and 40-80% saturation. After dialysis, they were then analyzed for the protein content and enzyme activity. The data are shown in Table 5.

Concentration by ammonium sulfate could increase a specific activity of crude dried malt rootlet from 0.14 unit/mg (non-concentrate) to 0.33 unit/mg (80% saturation) and 0.45 unit/mg (40-80 % saturation). The specific activity obtained at 40-80% saturation was higher than obtained at 80% saturation around 36%.

As these results, precipitation at 40-80% saturation was chosen for concentration of the crude extract of dried malt rootlets.

4. Preparation of 5′-GMP-rich yeast extracts

4.1 Determination of optimum yeast autolysis time for preparation of 5′-nucleotide-rich yeast extracts

The effect of yeast autolysis time on 5′-GMP content is shown in Figure 21. The lowest 5′-GMP content of around 0.1-0.3 %(w/w) was observed at 24 hr of autolysis. At 16 hr of autolysis, the 5′-GMP content was decreased with increasing of enzyme incubation time. For example, at enzyme concentration 100 unit/100 mL of yeast extract (5% w/v solids) the 5′-GMP content was decreased from 0.76 % (w/w) (8 hr of enzyme incubation) to 0.62 and 0.31 % (w/w) at 14 and 20 hr of enzyme incubation, respectively. The highest 5′-GMP content of around 0.7-0.9 % (w/w) was obtained at 8 hr of autolysis with 14 hr of enzyme incubation.

Samples	Enzyme activity (unit/mL)	Protein content (mg/mL)	Volume (mL)	Total activity (unit)	Total protein (mg)	Yield (%)	Specific activity (unit/mg)	Fold of purification
Crude malt rootlet extract (control)	3.25 ± 0.06	23.05 ± 0.91	100.00	325.00	2305.20	100.00	0.14	1.00
Concentrated crude malt rootlet extract (80 % saturation)	8.26 ± 0.09	24.66 ± 0.52	11.87	98.05	292.71	30.17	0.33	2.36
Concentrated crude malt rootlet extract (40-80 % saturation)	9.75 ± 0.14	21.65 ± 0.75	8.11	79.07	175.58	24.33	0.45	3.21

Table 5 Enzyme concentration of crude dried malt rootlet extract at 80 % saturation and 40-80% saturation of ammonium sulfate

Considering these results, It can be conculded that increasing of yeast autolysis time trend to decrease the 5′-GMP content (%w/w) in yeast extract. Therefore, the optimum autolysis time could be 8 hr or less.

4.2 Determination of optimum enzymatic hydrolysis time for preparation

Figure 21 also shows the effect of enzyme incubation on 5′-GMP content. At 8 hr of autolysis, 100 unit/100 mL yeast extract (5%w/v solids), the 5′-GMP content was slightly increased from 0.71 %(w/w) at 8 hr of enzyme incubation to 0.94 %(w/w) at 14 hr of incubation and decreased to 0.82 %(w/w) at 20 hr of enzyme incubation. At 16 hr of autolysis, also at 100 unit/100 mL yeast extract (5% w/v solids), the 5′-GMP content were gradually decreased from 0.77 to 0.65 and 0.33 % (w/w) at 8, 14 and 20 hr of enzyme incubation. At 24 hr of autolysis, the 5′-GMP content nearly constant. They were 0.28, 0.27 and 0.25 % (w/w) at 8, 14 and 20 hr of enzyme incubation, respectively.

According to these results, it can be concluded that shorter of enzymatic hydrolysis time should be preferable than longer time. The optimum enzyme hydrolysis time could be 8 hr or less.

4.3 Determination of optimum enzyme concentration for preparation

Figure 21 also shows the effect of enzyme concentration on 5′-GMP content in yeast extract. At 8 hr of autolysis, the 5′-GMP content was increased with

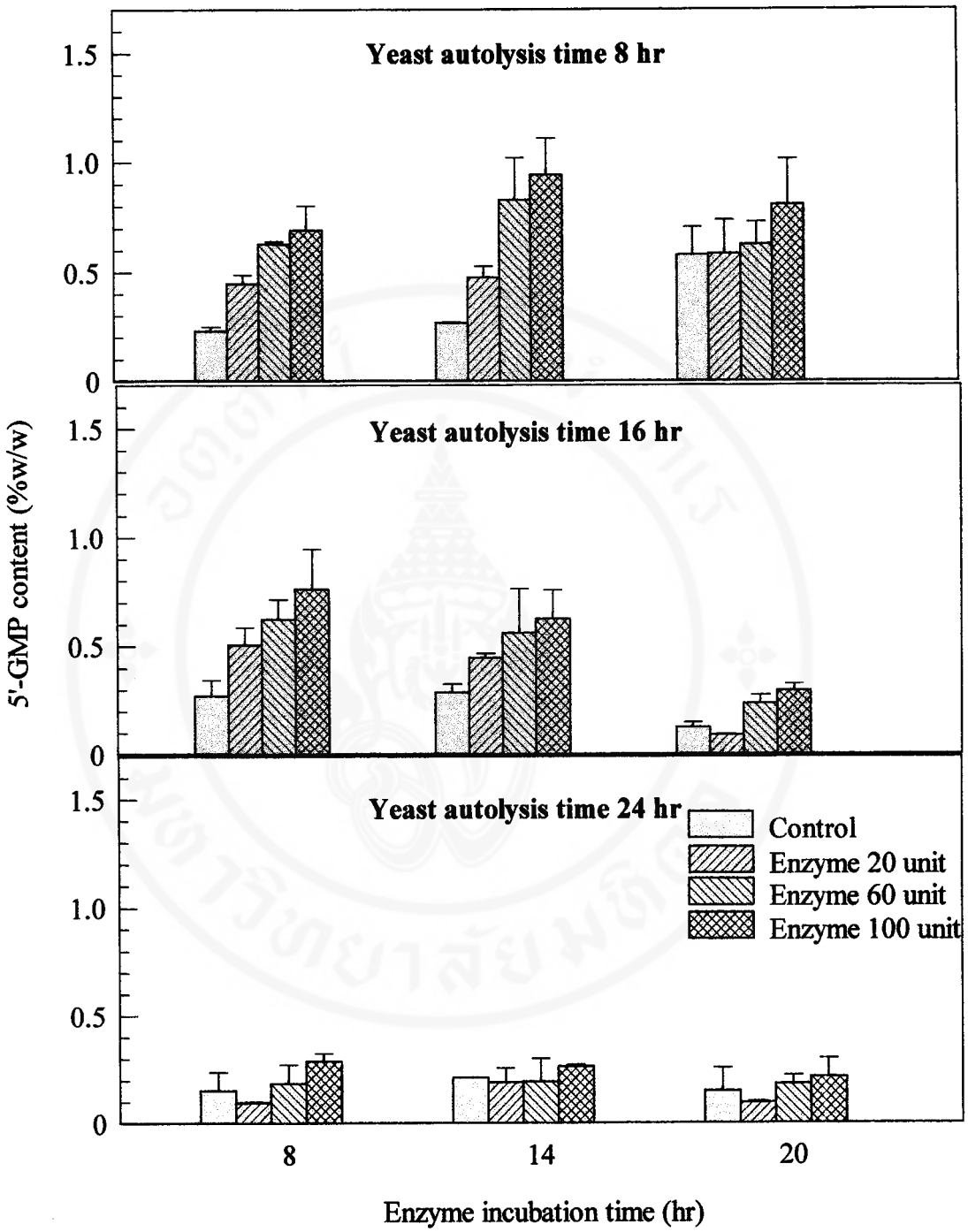


Figure 21 Effect of yeast autolysis time, phosphodiesterase incubation period and enzyme concentration on 5'-GMP content in yeast extract. Yeast suspension (15% w/v solids) was autolyzed at 50°C, pH 5 for 8, 16 and 24 hr. Yeast autolysates, pH 6.2-6.4 were then incubated at 90°C for 2 hr. The yeast extract (5%w/v solids) thus obtained were incubated with various concentration of phosphodiesterase at 60 °C pH 6.2-6.4. The 5'-GMP content was assayed after 8, 14 and 20 hr of incubation.

increasing of enzyme concentration at every period of enzyme incubation. At 16 and 24 hr of autolysis were also gave the same trend. According to these results, these profiles implied that it was remarkably possible to raise the 5′-GMP content in yeast extract if the enzyme concentration was increased.

From those conclusions of all parameters; autolysis time, enzyme hydrolysis time and enzyme concentration, they were used as preliminary data for further study. In subsequent study, the yeast autolysis times were 4, 8, 12, 16 and 20 hr. The enzymatic hydrolysis times were 2, 8, 14, 20, 26 and 44 hr. The procedure was carried out by the same conditions as previous study except the enzyme concentration was fixed at 100 unit per 100 mL yeast autolysate. The results of these experiments are displayed in Figure 22.

Considering a effect of enzyme incubation on 5′-GMP content found that mostly of 5′-GMP content was inclined at initial of enzyme incubation and then continuously decreased until terminated the time. The high 5′-GMP content was observed from 8-12 hr of autolysis. At 8 hr of autolysis, the 5′-GMP content were intently inclined from 0.55 to 0.68 and 0.86 % (w/w) at 2, 8, and 14 hr of enzyme incubation and then continuously declined to 0.80, 0.52 and 0.46 % (w/w) at 20, 26 and 44 hr of enzyme incubation, respectively. At 12 hr of autolysis, the 5′-GMP content were 0.54, 0.86, 0.83, 0.75, 0.63 and 0.44 %(w/w) at 2, 8, 14, 20, 26 and 44 hr of enzyme incubation, respectively. It was found that the high 5′-GMP content was obtained at a range of 8-14 hr of enzyme incubation. For 4, 16 and 20 hr of autolysis, the highest 5′-GMP content obtained from each autolysis time were 0.63, 0.54 and 0.43 % (w/w), respectively. They were also obtained at a rang of 8-14 hr of enzyme

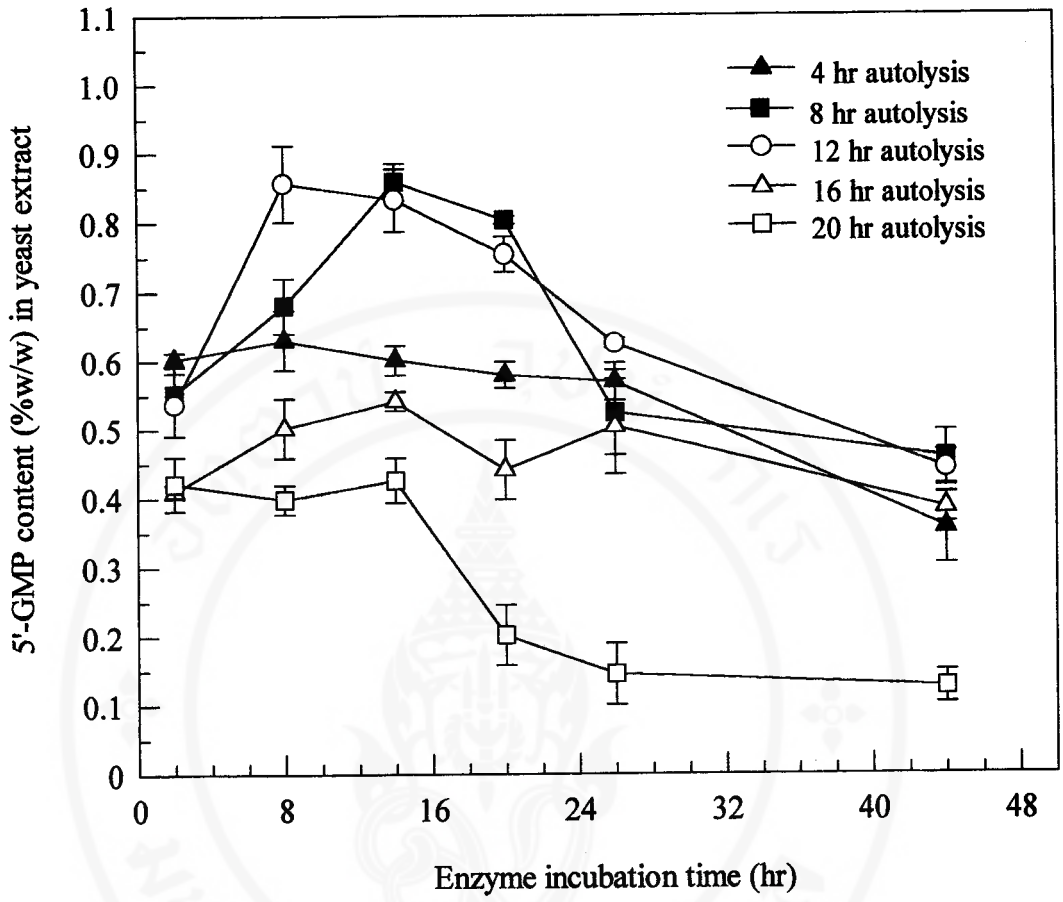


Figure 22 Effect of yeast autolysis time and phosphodiesterase incubation period on 5'-GMP content of non-debittered spent brewer's yeast extract. Yeast suspension (15 % w/v solids) was autolyzed at 50°C, pH 5, for various periods. The yeast autolysates thus obtained were adjusted to pH 6.2-6.4 and incubated at 95 °C for 2 hr in a stroke water bath operating at 200 strokes / min. After centrifugation, the yeast extract (5% w/v solids) were incubated with concentrate malt rootlet extract at enzyme concentration of 100 units per 100 mL of yeast extract. The mixture was adjusted to pH 6.2-6.4, incubated at 60°C for 2, 8, 14, 20, 26 and 44 hr in a stroke water bath operating at 200 strokes/min.

incubation. According to these results, it can be concluded that the optimum yeast autolysis time for preparation process of 5′-nucleotide-rich yeast extracts was 8 to 12 hr and the optimum enzyme hydrolysis time was 8 to 14 hr.

5. Preparation of 5′-GMP-rich yeast extracts from debittered spent brewer's yeast

Both intact brewer's yeast and extracts thereof are bitter, which is not desirable for application in food. Hence debittering of the yeast is necessary to obtain the minimum or absence of bitterness in the final product. The spent brewer's yeast from beer production was debittered by alkaline washing before using as raw material in this experiment. The procedure was performed using the same as previous experiment expect the enzyme incubation time was carried out at 0, 1, 2, 4, 8, 14, 20 and 20 hr. The results are shown in Figure 23.

The results show a trend similar to non-debittered cells. The highest 5′-GMP contents (ca. 0.7 % w/w) were observed with 8-12 hr of autolysis , lowest content of 5′-GMP (ca. 0.4%w/w) was observed at 20 hr of autolysis. Comparison of 5′-GMP in yeast extract between non-debittered and debittered yeast extract found that the 5′-GMP content of debittered brewer's yeast extract was less than non-debittered brewer's yeast extract approximately 0.1% (w/w) by average. It can be concluded that debittering can reduce the 5′-GMP content in yeast extract but on a low level.

In all cases, the 5′-GMP level began to decrease after a certain period of incubation of both debittered and non-debittered brewer's yeast extract. For instance;

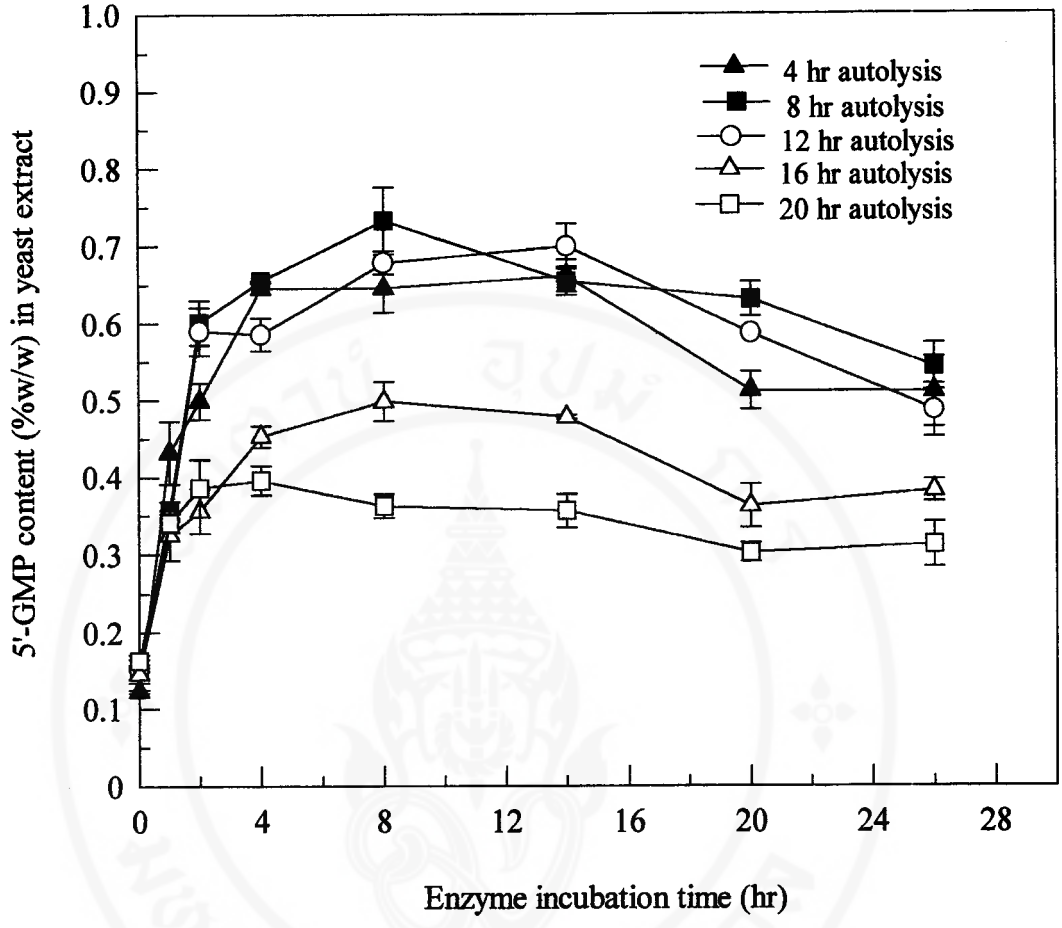


Figure 23 Effect of yeast autolysis time and phosphodiesterase incubation period on 5'-GMP content of debittered spent brewer's yeast extract. Yeast suspension (15% w/v solids) was autolyzed at 50°C, pH 5, for various periods. The yeast autolysates thus obtained were adjusted to pH 6.2-6.4 and incubated at 95°C for 2 hr in a stroke water bath operating at 200 strokes/min. After centrifugation, the yeast extract (5% w/v solids) were incubated with concentrate malt rootlet extract at enzyme concentration of 100 units per 100 mLof yeast extract.The mixture was adjusted to pH 6.2-6.4, incubated at 60 °C for 0, 1, 2, 8, 14, 20 and 26 hr in a a stroke water bath operating at 200 strokes/min.

at 8 hr of debittered yeast autolysis, the 5′- GMP content was decreased from 0.73 to 0.65, 0.63 and 0.54 % (w/w) at 8, 14, 20 and 26 hr of enzyme hydrolysis time, respectively. This may be due to the unstability of 5′-GMP under the enzyme hydrolysis condition and/or the destruction of 5′-GMP by 5′-nucleotidase present in the crude enzyme preparation. It was attempted to inactivate the latter by pre-treatment the crude malt rootlet extract.

6. 5′- Nucleotidase inactivation

6.1 Determination of suitable conditon for pre-treatment malt rootlet extract

To proof those of assumptions, the 5′-nucleotidase inactivation were studied. Many of pre-treatment conditions were chosen form literatures which mostly performed by heating or adding inhibitor. These study conditions were heating crude malt rootlet extract at 63°C for 1 hr, 72°C for 5 min, 72°C for 5 min along with adding inhibitor (0.005 M $Zn.Ac_2.H_2O$), 85°C for 7 and 30 min, 95°C for 5 min, 100°C for 10 and 30 min, non-pretreatment enzyme as a reference. The 5′-GMP solution without enzyme addition was incubated for stability test. The result is displayed in Figure 24.

The remaining 5′-GMP of 5′-GMP solution without malt rootlet extract addition was nearly stable during incubation. In contrast, the 5′-GMP solution with non-pretreated crude extract (reference) showed a sharply decreased of 5′-GMP at the initial of reaction and then slowly decreased up to the end of reaction. The lowest remaining 5′-GMP of reference was observed with 20 hr of incubation (84.50 %).

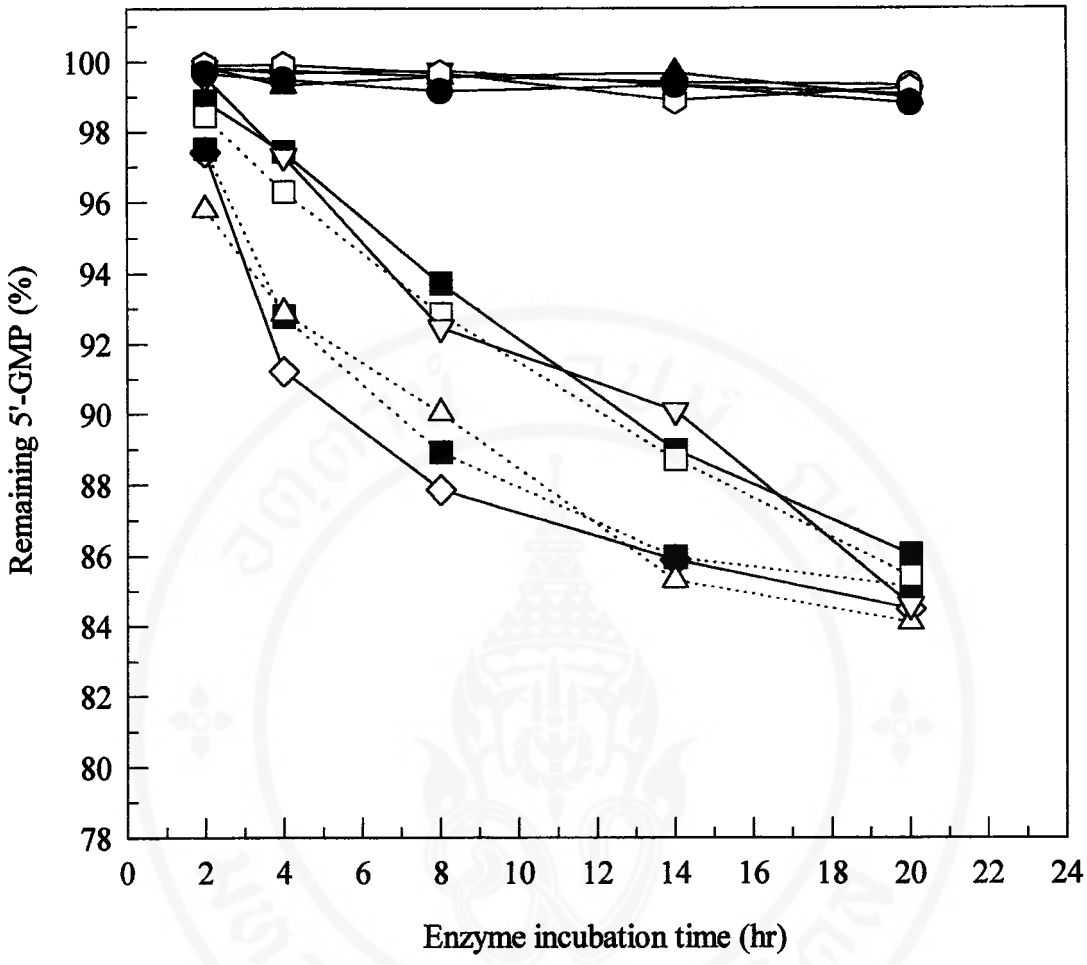


Figure 24 Effect of pre-treated malt rootlet extract on 5'- GMP. Solution of 0.1% (w/v) 5'-GMP 100 mL was mixed with 10 mL pre-treated crude malt rootlet extract at various conditions —◇— non heated malt rootlet extract (reference) ····■···· inhibitor (0.005 M $Zn.Ac_2.H_2O$), ····□···· heating at 72°C for 5 min, —■— inhibitor and heating at 72°C for 5 min, ····△···· 63°C for 1 hr, —▽— 85 °C for 7 min, —▼— 85°C for 30 min, —▲— 95°C for 5 min, —⬡— 100 °C for 10 min, —●— 100°C for 30 min, —○— 5'-GMP solution (without malt rootlet extract addition). The mixture was adjusted to pH 6.2 incubated in a stroke water bath operting at 200 strokes/min at 60 °C.

These results can be concluded that the 5′-GMP was stable under the enzyme hydrolysis condition and the 5′-nuleotidase exactly present in the crude malt rootlet extract and able degrade the 5′-GMP. For the results of harsh pre-treatment conditions; heating at 100°C for 30 and 10 min, 95°C for 5 min and 85°C for 30 min, all 5′-GMP slightly declined throughout the reaction (0.1 to 1.2 %). This shows that those of severe conditions able to inactivate the 5′-nucleotidase activity.

For the rest of data, the lowest remaining 5′-GMP was obtained from heat pre-treat at 63°C for 1 hr with 20 hr of incubation (84 %). Considering the remaining 5′-GMP at 8 hr of enzyme incubation which was the optimum enzyme hydrolysis time of preparation process, the remaining 5′-GMP of pre-treatment by adding inhibitor, heating at 63°C for 1 hr, 85°C for 7 min, 72°C for 5 min, 72°C for 5 min along with adding inhibitor were 88.92, 90.04, 92.47, 92.85 and 93.71%, respectively. As these data, the remaining 5′-GMP of pre-treatment with inhibitor ($Zn.Ac_2.H_2O$) addition was less than the reference approximately 1.0-1.50%. These results implied that adding $Zn.Ac_2.H_2O$ seemingly did not promote the 5′-nucleotidase inactivation. In common with adding $Zn.Ac_2.H_2O$ along with heating at 72°C for 5 min, the remaining 5′-GMP was similar to only heating at 72°C for 5 min. These results also confirm a previous conclusion.

From all of pre-treatment, heating at 85°C for 7 min seem to be the most suitable condition for pre-treatment cause gave the highest remaining 5′-GMP. However, the 5′-phosphodiesterase activity of malt rootlet extract may be destroyed at

those of pre-treatment temperatures. The 5′-phosphodiesterase activity of the extract heated at 85°C at various time intervals was investigated. These results found that the 5′-phosphodiesterase activity was almost destroyed with minimum heating time (5min), Table 6. According to these results, this condition unable to use as a pre-treatment condition.

In contract, the relative of remaining 5′-phosphodiesterase activities of heat pre-treat at 63°C for 1 hr and 72°C for 5 min was relatively constant (Table 6). Therefore, 72°C for 5 min was chosen as a heat pre-treat condition.

Heat pre-treat conditions	Relative remaining of 5′-phosphodiesterase activity (%)
63°C for 1 hr	94.27 ± 2.44
72°C for 5 min	96.19 ± 0.98
85 °C for 5 min	4.87 ± 1.06

Table 6 Relative remaining of 5′- phosphodiesterase activities of concentrated crude extract after pre-treatment by heating at 63°C for 1 hr, 72°C for 5 min and 85°C for 5 min.

6.2 Determination of optimum pH for concentrated malt rootlet extract

6.2.1 Optimum pH of 5′-phosphodiesterase activity

From previous conclusion, heat pre-treat at 72°C for 5 min was chosen as the suitable condition for 5′-nuclotidase inactivation. Nevertheless, the decreasing of 5′-GMP was still present. To improve this study, heating temperature and heating time were chosen as parameters for further study.

Before all else, to retain the highest 5′-phosphodiesterase activity during inactivation, the optimal pH of 5′-phosphodiesterase activity was investigated. The pH of concentrated rootlet extract ranged from 4 to 8.0. The results are shown in Figure 25. It was found that the highest 5′-phosphodiesterase activity was observed with pH 6.0 to 6.8. They were not statistically significant difference ($P > 0.05$). The pH lower or higher than this interval, the enzyme activity was gradually decreased.

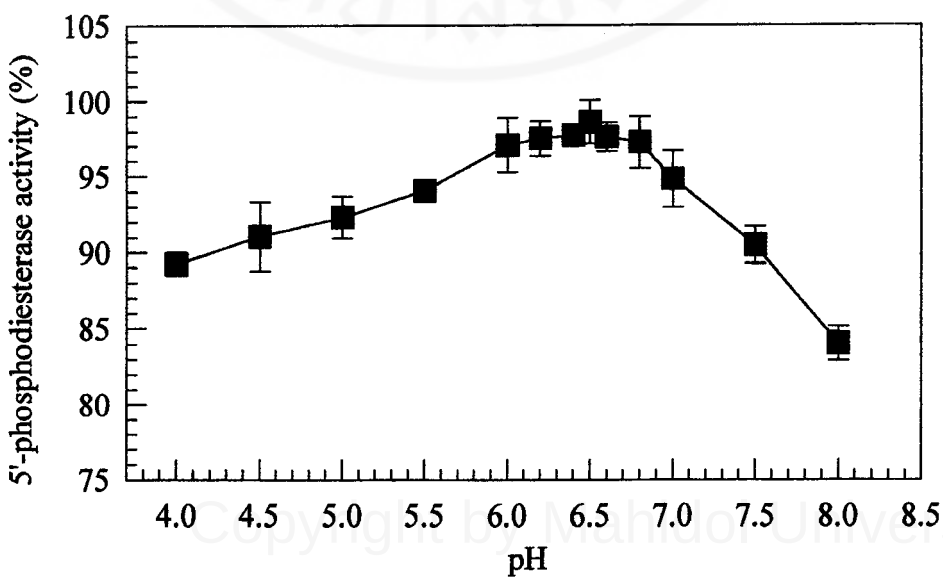


Figure 25 Effect of pH of concentrated crude malt rootlet extract on 5'-phosphodiesterase activity.

6.2.2 Determination of optimum heating time and temperature for 5′-nucleotidase inactivation

Heating time and temperature were chosen as the parameters for 5′-nucleotidase inactivation. The malt rootlet extract was heated at various times and temperatures. The temperatures ranged from 60 to 80°C and heating times ranged from 2 to 180 min. The results are displayed in Figures 26, 27.

Figure 26 shows the effect of pre-heating the crude phosphodiesterase preparation as a function of heating time and temperature upon incubation with pure 5′-GMP. The results indicate that the higher of heating temperature and/or the longer heating time, the higher the remaining 5′-GMP. The highest residual level of 5′-GMP (94%) was observed at pre-treatment temperatures of 80°C, 75°C and 70°C for 15, 30 and 60 min, respectively, whereas a lower value (91%) was obtained at a temperature of 60°C for 60 min (Figure 26). These results may imply that increasing the temperature for pre-treatment resulted in a more effective inactivation of 5′-nucleotidase. However, the heat treatment also markedly affected the 5′-phosphodiesterase activity as shown in Figure 27. More than 80 % and 50 % of the activity was lost at 80°C for 2 min and 75°C for 15 min, respectively. On the other hand, heating the enzyme at lower temperatures and longer periods, *e.g.* 65°C for 120 min or 60°C for 180 min, resulted in less than 20 % loss of 5′-phosphodiesterase activity (Figure 27).

From these data it can be concluded that the best compromise in terms of high residual 5′-phosphodiesterase activity and 5′-nucleotidase inactivation was treatment at 65°C for 30 min or 70°C for 7 min (results not statistically different at $P<0.05$), which was used for the following experiments.

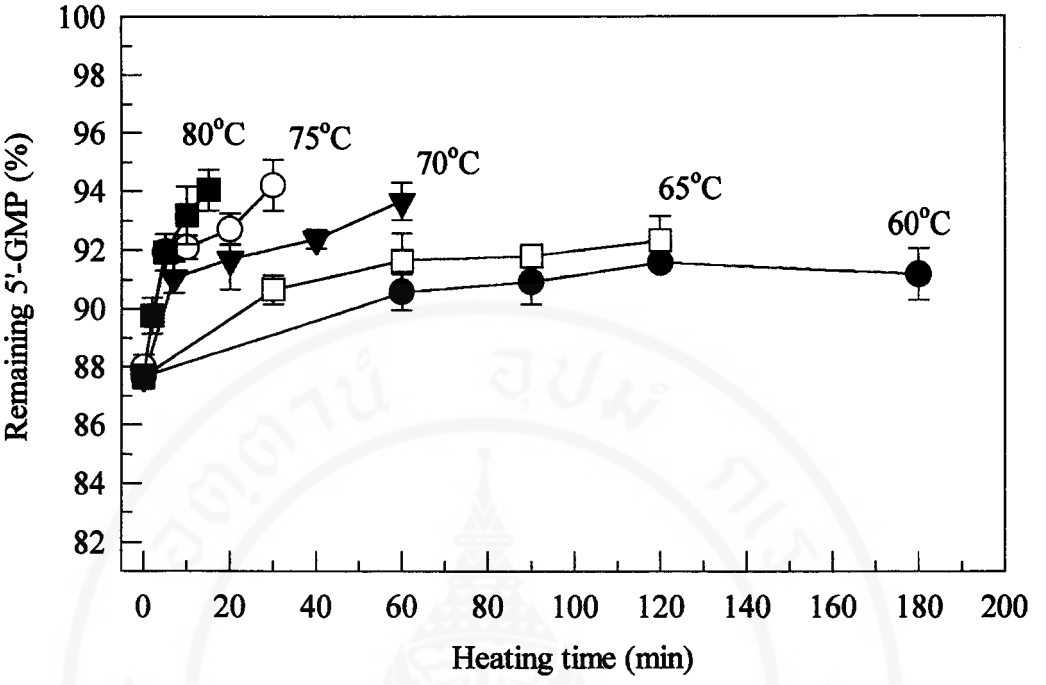


Figure 26 Effect of heat pre-treated malt rootlet extract on 5'-GMP. Crude malt rootlet extract was heated at various temperatures and periods, adjusted to 60°C / pH 6.2 and incubated with 0.1 % (w/v) pure 5'-GMP for 8 hr. Residual 5'- GMP was then assayed.

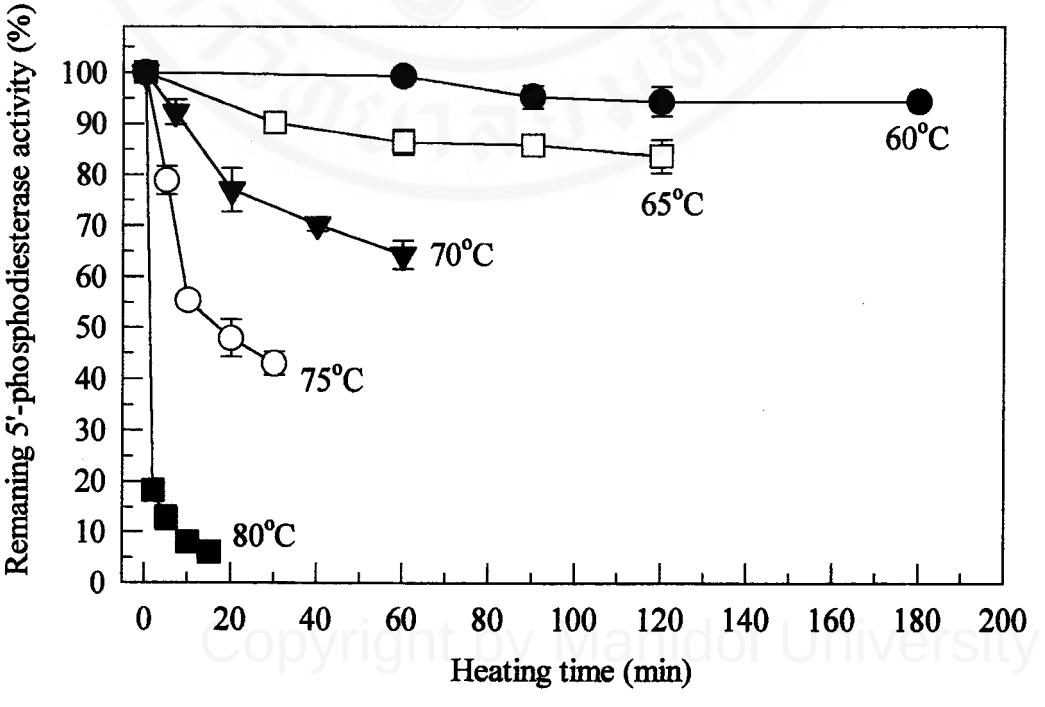


Figure 27 Effect of heat pre-treatment on 5'-phosphodiesterase activity in crude malt rootlet extract.The extracts were heated at the temperatures and periods indicated, cooled down, followed by assay of 5'-phosphodiesterase

A 5′-GMP solution without addition of enzyme was stable for at least 26 hr (data not shown).

6.3 Using heat pre-treated extract in preparation of yeast extracts

In order to confirm any effects of 5′-nucleotidase inactivation on 5′-GMP formation, a heat-treated malt rootlet was used in the preparation of 5′-GMP rich yeast extracts. From Figure 28, the 5′-GMP content of yeast extract incubated with heated malt rootlet extract was slightly higher than without heating, with an average increase of 7 %. The highest 5′-GMP contents (0.75-0.78 %w/w) were obtained with 8-14 hr of incubation. As these results, it can be concluded that heat pre-treated malt rootlet extract able improved the preparation of 5′-GMP-rich yeast extract. Nevertheless, it was still improve in a low level.

7. Effect of phosphodiesterase concentration on 5′-GMP level

The results of previous experiment (Figure 21) indicate that 5′-GMP content of yeast extract increased with increasing phosphodiesterase levels. This was further studied to find the phosphodiesterase concentration resulting in the highest level of 5′-GMP. The result is shown in Figure 29.

From the Figure, the 5′-GMP content was sharply increased with increasing of enzyme concentration and after that the 5′-GMP content was leveled off. Increasing the phosphodiesterase concentration to 160 unit per 100 mL yeast extract increased 5′-GMP level to a plateau of ca 0.93 % (w/w). The enzyme concentration more than

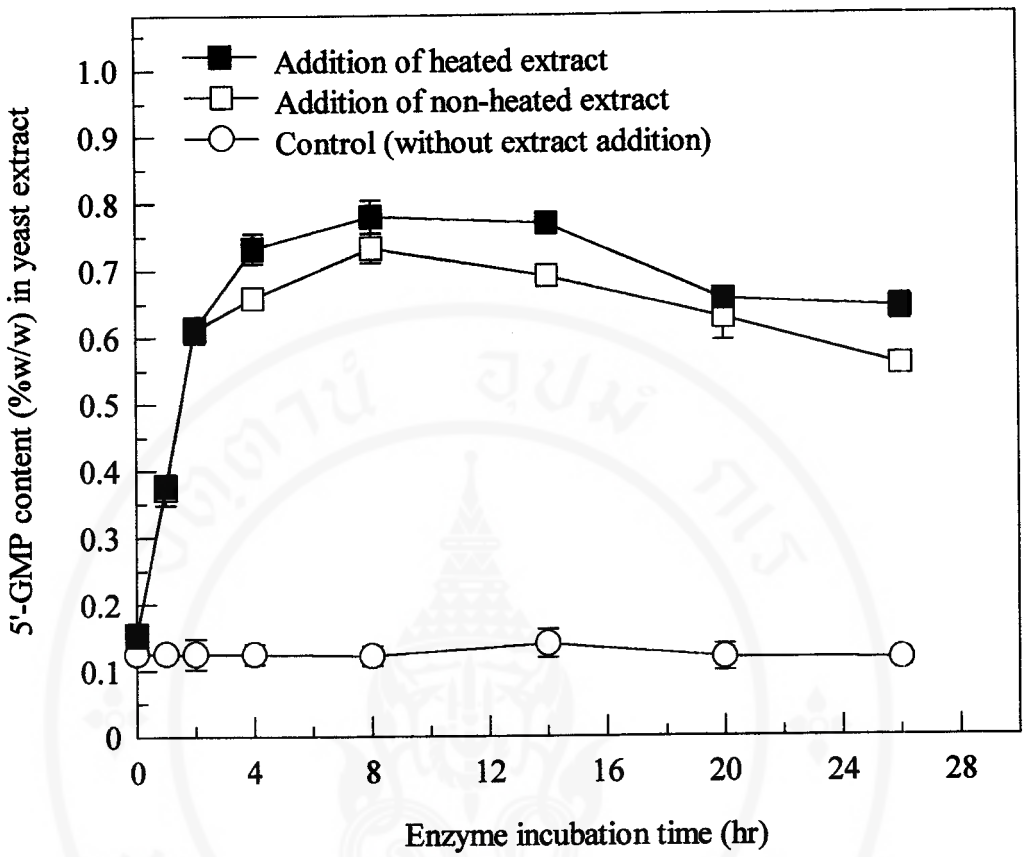


Figure 28 Effect of pre-heating (70°C for 7 min) crude malt rootlet extract on 5'-GMP content in debittered yeast extract as a function of incubation time. Debittered yeast extract was obtained by autolysis for 8 hr. In addition, incubation was performed with non-heated extract. In both cases, enzyme concentration 100 units / 100 mL yeast extract (5% w/v solids) was used. A sample without malt rootlet extract was used as a control.

this level did not improved the 5′-GMP content in yeast extract.

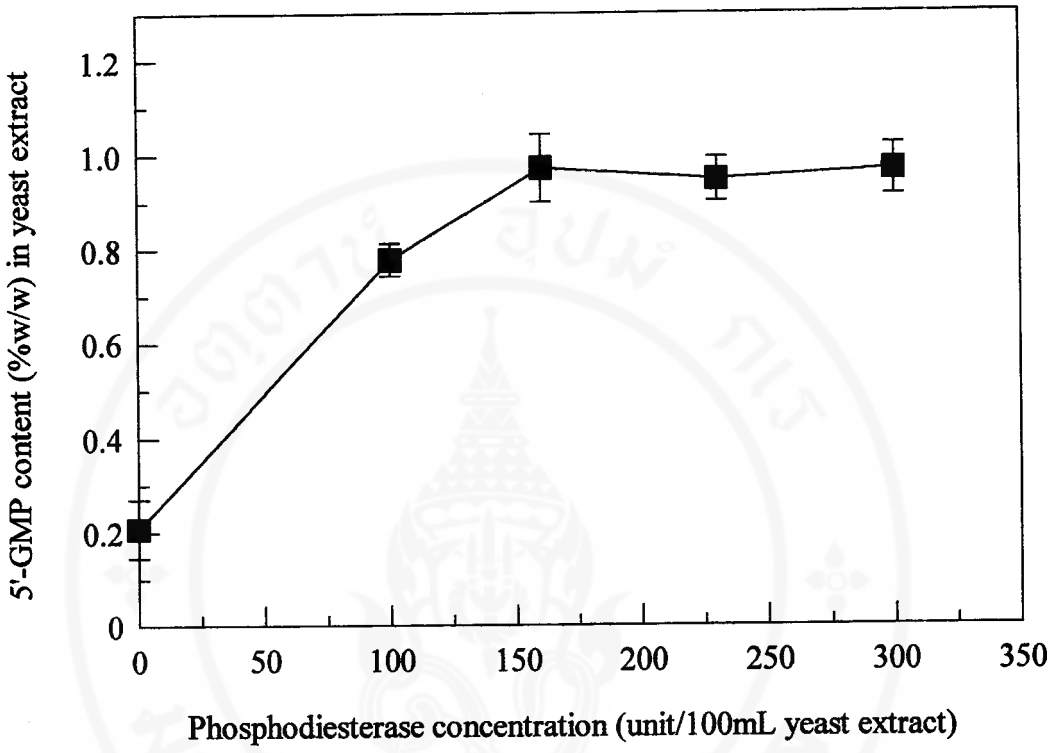


Figure 29 Effect of phosphodiesterase concentration on 5'-GMP content of debittered spent brewer's yeast extract. Debittered yeast extract (15% w/v solids) was obtained by autolysis for 8 hr, followed by incubation with various amounts of pre-heated (70°C for 7min) malt rootlet extract for 8 hr.

8. Cost estimation of 5′-GMP-rich yeast extracts

The total cost of a large-scale process is usually strongly dependent on the production volume. The calculations shown in Table 7 were modified from those described by Verduyn, 1997 [83] based on a batch production of 500 kg of 5′-GMP-rich yeast extract. The spent brewer's yeast (18 % solids) was debittered and autolyzed for 8 hr. After the yeast extract was incubated with pre-heated concentrated crude extract for 8 hr, it was concentrated and dried by spray-drying then obtained the yeast extract powder with approximately 5-7 % moisture content. The cost estimation of 5′-GMP-rich yeast extracts was about 423 baht/kg. From this estimation, the major cost of process from ammonium sulfate, used for precipitation of crude malt rootlet extract. It was approximately 51.6 % of total operating cost or 77.8 % of raw material. The other major cost came from utilities like process water and steam. These cost ca 30 % of total operating cost. The amount of process water was mostly used for removal of salt from precipitated crude extract. The steam was mostly used for spray-drying.

From a good-standard commercial food-grade extract like Gistex (produced from baker's yeast) from Gist-brocades is sold for approximately 500 Baht/kg in Thailand. It contains about 38 % w/w salt (as NaCl). Neglecting the price of the salt, the cost of 1 kg of this yeast extract then amounts to approximately 800 Baht/kg. Comparison of this cost, the 5′-GMP-rich yeast extracts was about a half of this cost. However, this cost estimate was only base on a low volume of production. The other cost factors have to consider for a large volume of production.

Table 7 Cost estimation of 5′-GMP-rich yeast extracts production

		Unit cost (Baht/unit)	Cost/ batch [a]	Total cost
1) Raw materials				
- Dried malt rootlets (95% solids)	37,467.4 kg	0.5 Baht/kg	18,733.8	
- Spent brewer's yeast (18 % solids)	10,869.5 kg	1 Baht/kg	10,869.5	
- Sodium carbonate (debittering)	43.0 kg	12 Baht/kg	516.0	
- Sulphuric acid (98%) (pH control)	34.4 kg	7 Baht/kg	240.8	
- Sodium hydroxide (pH control)	16.7 kg	18 Baht/kg	300.8	
- Ammonium sulfate (crude extract precipitation)	11,489.2 kg	9.5 Baht/kg	109,145.5	
- Packaging bags	(10 kg/bag)	7 Baht/pag	350.0	
2) Utilities				
- Process water	342.4 m^3	100 Baht/m^3	34,239.0	
- Steam	10.1 ton	3,000 Baht/ton	30,205.5	
- Cooling water	54.3 m^3	50 Baht/m^3	2,717.2	
- Electricity	1,184.8 kW-h	3.5 Baht/kW-h	4,146.8	
				211,464.8 [b]

[a] Unit cost per 500 kg of 5′-GMP-rich yeast extracts

[b] This cost is for production of 500 kg of 5′-GMP-rich yeast extracts, it is approximately **423 Bath/kg**

9. Determination of RNA content in samples of preparation of 5′-GMP-rich yeast extracts

Determination of RNA content in samples of preparation process of 5′-GMP-rich yeast extracts was divided into 3 sections; raw material pre-treatment, autolysis, RNA extraction and enzyme incubation. RNA content in samples of each section was determined by absorbency method. The commercial yeast extracts were also determined for the RNA content. The results are displayed in Table 8-10.

For the section of raw material pre-treatment, RNA content in debittered brewer's yeast cells was slightly higher than those of non-debittered brewer's yeast cells. For autolysis and RNA extraction section, RNA content in yeast extract after RNA extraction (11.81 %w/w) was not different from before extraction (11.09 %w/w). At the same way, RNA content in cell debris before RNA extraction (5.24 %w/w) was similar to after extraction (4.57 %w/w). From statistic test, the RNA content in debittered and non-debittered yeast cells as well as RNA content in yeast extract and cell debris of both before and after RNA extraction were not statistically significant different ($P > 0.05$). It can be concluded that debittering was not effect on RNA content in yeast cells. In common with RNA extraction, it was slightly increase the efficiency of preparation owing to a minute increment of RNA content in yeast extract and a minute decrement of RNA content in cell debris.

For enzymatic hydrolysis section (Table 9), yeast extract obtained at 0-20 hr of enzyme incubation was determined for remaining RNA content and 5′-GMP content. From the Table 8, remaining RNA content was considerably decreased from 10.79 % (w/w) at 0 hr of enzyme hydrolysis to 5.82 % (w/w) at 2 hr of enzyme hydrolysis and then nearly constant at 4 hr (4.7 % w/w) throughout the run.

Table 8 RNA content in samples of 5′-GMP-rich yeast extracts

Samples		RNA content* (%w/w)
Raw material pre-treatment	Non-debittered brewer's yeast cells	8.03 ± 0.12 [a**]
	Debittered brewer's yeast cells	9.10 ± 0.34 [a]
Autolysis (8hr) and RNA extraction	Yeast extract (before RNA extraction)	10.96 ± 0.25 [a]
	Yeast extract (after RNA extraction)	11.88 ± 0.57 [a]
	Cell debris (before RNA extraction)	5.24 ± 0.38 [a]
	Cell debris (after RNA extraction)	4.57 ± 0.47 [a]

* Mean ± standard deviation (SD) with n = 6

** Means values within the same rows with similar superscripts are not significantly different (P > 0.05)

For 5′-GMP content (Table 9), it was continuously increased from 0.17 % (w/w) at 0 hr to 0.79 % (w/w) at 8 hr of enzyme hydrolysis and then decreased to 0.72 % and 0.68 % (w/w) at 14 and 20 hr of enzyme hydrolysis, respectively.

For commercial yeast extracts (Table 10), the maximum RNA content obtained from Maxaravor Rye-B Powder (8.85 %w/w) followed by Maxarome standard powder (8.44 %w/w), Gistex AGGL (7.57 %w/w) and Quest (7.38 %w/w), respectively.

Table 9 RNA content and 5′-GMP content in yeast extract (8 hr of autolysis) at various enzyme hydrolysis times.

Enzyme hydrolysis time (hr)	Remaining RNA content* (%w/w)	5′-GMP content (%w/w)
0	10.79 ± 0.36	0.17 ± 0.02
2	5.82 ± 0.80	0.56 ± 0.07
4	4.83 ± 0.28	0.71 ± 0.01
8	4.78 ± 0.36	0.79 ± 0.09
14	4.76 ± 0.16	0.72 ± 0.01
20	4.70 ± 0.57	0.68 ± 0.05

Table 10 RNA content in commercial yeast extract

Commercial yeast extracts	RNA content (%w/w)*
Maxarome standard powder	8.44 ± 0.28 [a]
Quest	7.38 ± 0.33 [b]
Gistex AGGL	7.57 ± 0.24 [b]
Maxavor Rye-B Powder	8.85 ± 0.18 [c]

* Mean ± standard deviation (SD) with n = 6

[a, b, c] Means values within columns with different superscripts are significantly different ($P \leq 0.05$)

10. Study of 5′-GMP produced from pure substrate RNA

The study of conversion efficiency from pure substrate RNA to 5′-GMP by crude malt rootlet extract may be was used for expectation of 5′-GMP level occurred in preparation process. For this study, RNA was prepared as starting substrate solution having concentration were 0.5, 1.0 and 1.5 % (w/v) respectively. They were incubated with crude dried malt rootlet extract at various enzymatic hydrolysis time. The result is shown in Figure 30.

At 0.5 % (w/v) RNA solution, 5′-GMP was gradually increased in a low level with increasing of enzyme incubation time. From statistic test, the 5′-GMP from 4 to 20 hr of incubation were not statistically significant different ($P > 0.05$). At 1.0 % (w/v) RNA solution, 5′-GMP was increased from 0 to 4 hr of incubation and then relatively constant. The 5′-GMP from 2 to 20 hr of incubation were not statistically significant different ($P > 0.05$). At 1.5 % (w/v) RNA solution, 5′-GMP was also intently increased from 0 to 4 hr and then leveled off. The RNA content from 4 to 20 hr were not statistically significant different ($P > 0.05$). As these results, it can be concluded that at 0.5-1.5 % (w/v) RNA concentration and enzyme hydrolysis time ranged from 4 to 20 hr, the 5′-GMP level were not different.

Figure 31 shows the relationship between concentration of starting substrate pure RNA and initial velocity of enzyme. The profile was sharply increased from RNA concentration of 0.5 % (w/v) to 1.0 % (w/v). After that, the increasing of the initial velocity was slightly decreased at RNA concentration 1.5 % (w/v). From the profile, it can be concluded that the initial velocity trend to increase with increasing of RNA concentration.

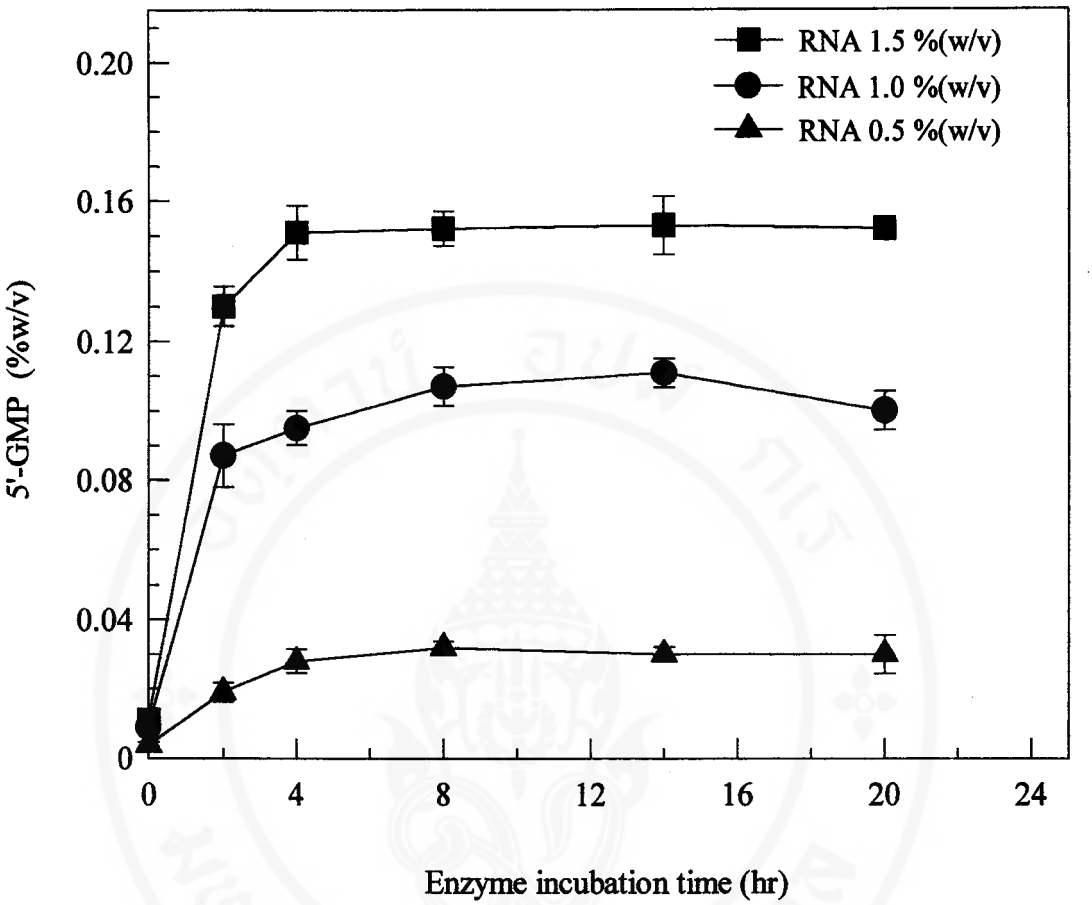


Figure 30 The 5'-GMP at various starting substrate RNA concentrations (from baker's yeast). RNA solution were mixed with heat pre-treated malt rootlet extract (70°C for 7 min) at a ratio of 100 mL of RNA solution and 100 units of enzyme concentration for 0, 2, 4, 8, 16 and 20 hr of enzyme incubation at 60 °C.

From this experiment, the RNA conversion to 5′-GMP was one of interesting data. It was calculated form the 5′-GMP content and the percentage of starting substrate RNA as shown in Figure 32. From this Figure, the RNA conversion to 5′-GMP at substrate RNA of 1.0 % and 1.5 % (w/v) was about twice of those obtained from substrate concentration of 0.5 % (w/v).

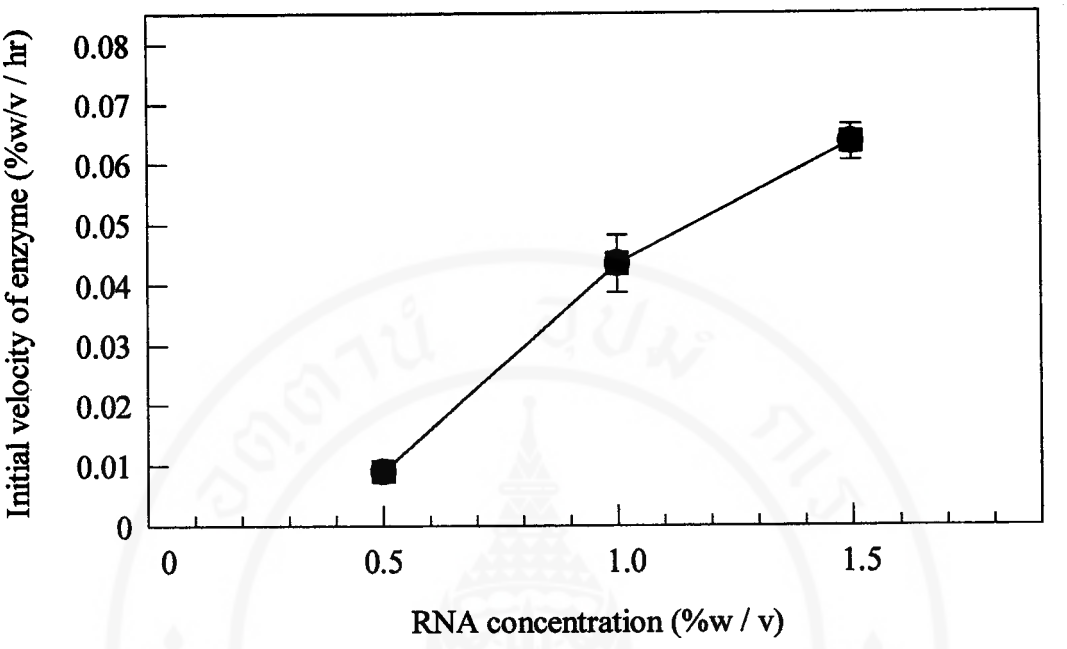


Figure 31 The relationship between concentration of starting substrate pure RNA (%w/v) and initial velocity of enzyme (%w/v / hr)

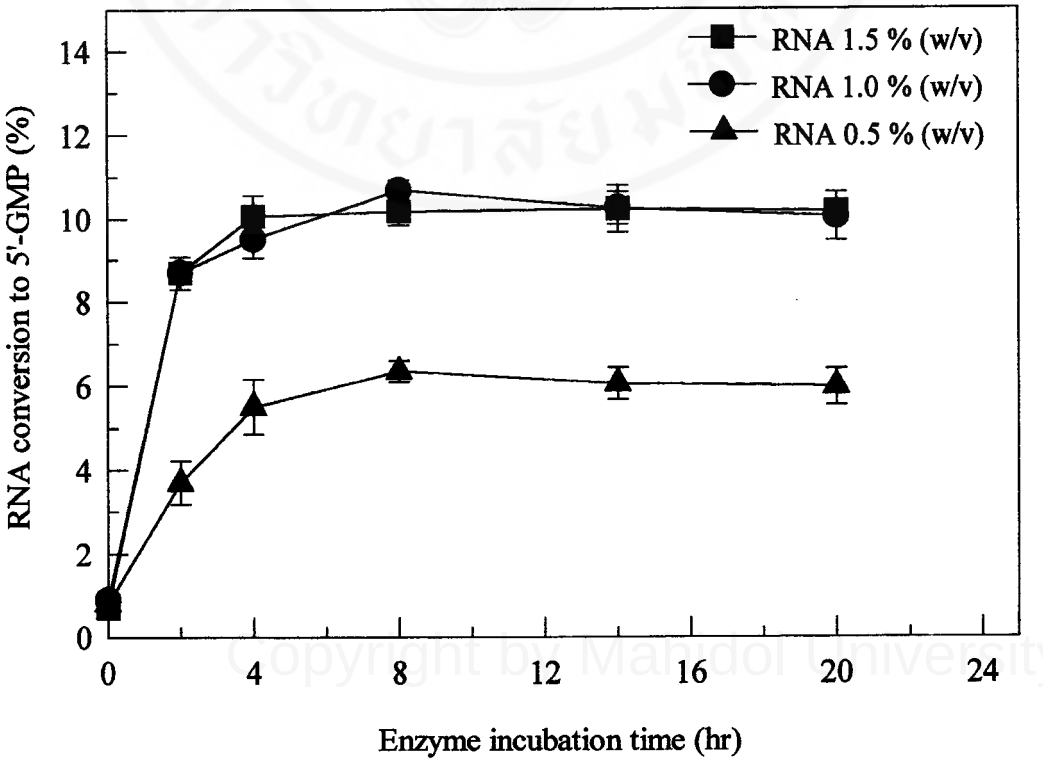


Figure 32 RNA conversion to 5'-GMP at various starting substrate RNA concentrations

CHAPTER V

DISCUSSION

1. Determination of optimum condition of high performance liquid chromatography (HPLC) for 5′-nucleotide analysis

The optimum condition of HPLC was studied base on the study of Zhao Jian and Fleet H G [81]. The elution was performed with gradient system comprised of two eluents. Eluent A was a solution of 0.05 M KH_2PO_4 adjusted to pH 5.45±0.02 with 0.05 M K_2HPO_4. Eluent B was 100 % methanol (HPLC grade). The column was a water μ Bondapak C_{18} 10 μm stainless-steel column (30 cm x 3.9 nm I.D). The absorbance detector operating at wavelength of 254 nm.

From the result of a standard mixture of nucleotides (Figure 12), the 5′-UMP eluted first followed by 5′-IMP at the same time of 5′-GMP, 5′-AMP, 3′-GMP and 2′-GMP. This elution order is in good agreement with elution order of standard mixture of nucleotides described by Zhao Jian and Fleet H G [81] and also by Fujinari M E and Manes J D (Gradient system; solvent A was 2.9 mL H_3PO_4/1L H_2O, pH 5.7. Sovent B was 2.9 mL H_3PO_4/1L 40% H_2O/60% MeOH, pH 5.9) [84].

The reason of this result can be described by Ramos LD and Schoffstall MA [85] who reported that compounds were eluted generally in the same order as their polarities and water solubilities. The more highly retained substances are those which are more hydrophobic. Guanine has one more polar substituent than adenine: this contributes to shorter retention time for guanine. The polarity order is 5′> 2′ >3′, so the elusion order was presented as this result. However, both of flavor enhancer

substances, 5′-IMP and 5′-GMP, were not separated. They were eluted as a one peak at this condition. This reason might be due to their similar properties as previously mentioned.

Attempts to improve the resolution of 5′-GMP and 5′-IMP separation by reduction of the flow-rate of the mobile phase was unsatisfied (Figure 13). It was not only slightly improved the resolution but also increased the amount of peak broadening seen.

The other factor that could probably be improving separation was the concentration of methanol in mobile phase. This factor has been described by Zhao Jian and Fleet H G [81] and Ramos LD and Schoffstall MA [85]. The results found that increasing the concentration of methanol in the mobile phase did not improve the resolution in the separation of 5′-GMP and 5′-IMP (Figure 14). In contract, it gave a poor resolution because those of peaks were shift closer with higher concentration of methanol. This result can be distinguish noticed from Figure 15, the concentration of methanol up to 2 % (v/v) was not effect on the separation. This result is in good agreement with the research described by Ramos LD and Schoffstall MA and Zhao Jian and Fleet H G [81].

Isocratic system has been reported for separation of some nucleotides, nucleosides and bases from biological extracts [86]. It was chosen as a comparative method of gradient system. This isocratic system was performed with one eluent. The eluent still was a solution of 0.05 M KH_2PO_4 adjusted to pH 5.45±0.02 with 0.05 M K_2HPO_4. The flow rate of eluent being maintained at 1.5 mL/min and performed at ambient temperature. Although from the advantage property of gradients system which are decrease the polarity of the system and speed up the elution of those

compounds which are retained the longest [87]. However, the result (Figure 17) found that the separation of both systems were not significantly different. The isocratic system was slightly better than gradient system. It was chosen for further study because of its simpler and easier to operate.

Within a chromatographic mode, the pH of the eluent plays a major role in determining the extent of dissociation of a solute [88]. The eluent pH 4.45-6.5, safety range of pH for operation, was studied and found that the best pH was 4.50-5.00. As the pH of eluent was incresed, the separation of 5′-GMP and 5′-IMP was decreased. At pH 6, they were eluted as a one peak.

In conclusion of this study, isocratic system was chosen as a condition for 5′-nucleotide analysis. It was perform with a solution of 0.05 M KH_2PO_4, pH 4.50-5.00. The flow rate was 1.5 mL/min at ambient temperature.

2. Comparison of 5′-phosphodiesterase of dried malt rootlets, fresh malt rootlets and fresh rice rootlets.

Numerous literature reviews have been reported about the 5′-phosphodiesterase enzyme, converts RNA to 5′-nucleotides, that cloud be extracted from various sources such as a snake venom, *Aspergillus niger* and malt rootlets [6]. Malt rootlets, a by product from malting industry, was an interesting source of 5′-phosphodiesterase because it could be easily available from commercial, cheap and also no doubt about safety of enzyme [4].

A comparison of 5′-phosphodiestease activity of crude rootlet extract of several types of rootlets, the highest activity was obtained from fresh malt rootlets, followed by dried malt rootlets and rice rootlets. Considering the specific activity

between fresh malt rootlets (0.095 unit/mg protein) and dried malt rootlets (0.066 unit/mg protein), the specific activity of fresh malt rootlets was reduced approximately 30 % when the rootlets was dried. It is doubtfully to consider that normally under drying process of malting industry or called kilning process, the enzyme activity should extremely destroyed under this process, but from these results only partial of enzyme activity in malt rootlets was destroyed. The reason of these results can be described by the review of Briggs DE *et al*, 1981 [81] who described that in kilning process the germinated barley has to be dried to a low moisture content by passing a flow of hot dry air throuhg a malt at various rates. Initially malt is normally dried in a rapid air flow at comparatively low temperature (50-60°C) and, when its moisture content has been reduced sufficiently, increase the temperature (65-75 °C) until the product was dried (moisture content 5-8%). Temperature within kiln is controlled rigidly to minimize the denaturation of malt enzyme during the drying process.

This result is in good agreement with the US patent described by Laufer L *et al*, 1969 [72] who reported that the enzyme activity is little or no loss if the rootlets or stems are dried, even if such drying is at relatively high temperature as is generally employed in kilning malt. Furthermore, the activity is retained in the rootlets for a number of years without any special storage precaution.

As these results, dried malt rootlets were therefore preferable source of 5′-phosphodiesterase for preparation of 5′-GMP-rich yeast extracts. For rice rootlets, although the enzyme activity of rice rootlets was nearly one time lower than fresh malt rootlets and also lower than dried malt rootlets. This data was at least used as a primary study for investigation of the source of 5′-phosphodiesterase in common plant of Thailand.

3. Preparation of 5′-GMP-rich yeast extracts

The parameters effecting 5′-GMP content in preparation process were yeast autolysis time, enzyme hydrolysis time and enzyme concentration. In this study, the preparation process was divided into 2 sections. In autolysis section, yeast suspension (15% solids content, pH 5) was incubated at 50°C for 8-24 hr. In enzyme hydrolysis section, yeast extract was mixed with concentrated rootlet extract at various enzyme concentration, adjusted to pH 6.2-6.4 and then incubated at 60°C for 8-20 hr. The result of yeast autolysis time on 5′-GMP content (Figure 21) can be concluded that 5′-GMP content was decreased with increasing autolysis time, the highest of 5′-GMP was obtained from the shortest period of yeast autolysis time (8 hr). The reason of this result might be described by report of Nagodawithana TW *et al* ,1995 [9] which described that during autolysis process, after the death of yeast the degradative enzymes inherently present within the cell, allowing the free degradative enzymes to indiscriminately attack their specific substrates caused the breakdown of the corresponding macromolecules to their basic units. In this process, RNA was also degraded by inherently degradative enzyme, nucleases, to their basic units. These nuclease convert RNA to 2′- and 3′- nucleotides, which unlike the 5′-nucleotides have no effect on flavor in food system [1].

As those report, the possible reason of this result might be due to at long period of autolysis time the RNA was more degraded to be smaller subunits than those of a short period of time, so that RNA at short autolysis time was remain higher than those of long autolysis time. Since the 5′-nucleotides were converted from RNA, therefore the 5′-GMP at 8 hr of autolysis time was higher than at 16 or 24 hr of autolysis time.

For the effect of enzyme hydrolysis time on 5′-GMP content, the result (Figure 21) shows that 5′-GMP content was increased at early period of incubation and then gradually decreased with increasing incubation time. From this study, the preferable time of enzymatic hydrolysis at various autolysis time was 8-14 hr of incubation. This seems to be in good agreement with literature data. For instance, the hydrolysis of the RNA in yeast extract by 5′-phosphodiesterase derived from malt roots is carried out for 2 to 20 hr of incubation at a temperature of 50 to 60 °C at pH 6.0-6.6 [4]. Laufer L *et al*, 1967 [71] also described about the production of 5′-nucleotides from RNA solution by seed parts extract that hydrolysis of 5′-phosphodiesterase is generally completed in about 1.5-5.5 hr at 60-70°C, pH 5.2-8.5. Potman *et al*, 1994 [64] also described about the method for preparation of yeast extract containing 5′-GMP and free amino acid. RNA degradation by malt rootlets under oxidizing condition was carried out at 65°C pH 5.6 for 12-24 hr and under anaerobic condition was carried out at 65°C pH 5.6 for 6 hr.

For further study, the enzyme concentration was fixed at 100 units/100mL of yeast extract. In autolysis section, autolysis time was varied from 4-20 hr. In enzyme hydrolysis section, the enzyme hydrolysis time was varied from 2-44 hr. The result of this experiment (Figure 22) was relatively corresponding to a result of previous study in that the 5′-GMP content was increased at early autolysis as well as enzyme hydrolysis and then gradually declined. The highest 5′-GMP content was still obtained at an autolysis time of 8-12 hr and an enzyme hydrolysis time of 8-14 hr. The reason of this result also can be described with those previous reviews by Nagadawithana, 1995 [9] as previously mentioned.

4. Comparison of 5′-GMP content between non-debittered and debittered spent brewer's yeast extract

Although spent brewer's yeast can be used as an important raw material to produce yeast extract using as food flavoring agent [3, 10, 12, 79] it is likely to contain undesirable flavor characteristics as a result of carry over of hop resins and beer solid from the brewery fermentation [17]. To obtain the minimum or absence of bitterness in final product, so that debittering is necessary for the production process. The hop resins present on the surface of the yeast cell can be solubilized by an alkaline wash [20-22].

A comparison of 5′-GMP content between non-debittered (Figure 22) and debittered brewer's yeast extract (Figure 23) found that their 5′-GMP profiles were not quite different, the optimum autolysis time was still 8 hr and the enzyme hydrolysis time was also 8 hr. The 5′-GMP content of debittered yeast extract was slightly less than non-debittered yeast extract by approximately 0.1% w/w. These results indicated that debittering brewer's yeast by alkaline wash was slightly influence on 5′-GMP content of yeast extract. The possible reason might be due to the bitterness substance from hop resins that created a bittering characteristic was only associated with the surface of yeast cell [19] and the alkaline wash capable removed the bitterness substance by eliminating hydrogen bonds and other weak forces of bitterness substance from the surface yeast cell wall [20]. Therefore, it was reasonably to conclude that alkaline wash hardly disrupted the 5′-GMP content of yeast extract. Nevertheless, it has been reported that debittering spent brewer's yeast by alkaline wash was sometimes reduces the final extract yield [3].

5. 5′-Nucleotidase inactivation

The observation from 5′-GMP profile of both non-debittered and debittered yeast extract found that the 5′-GMP profile was gradually reduced with increasing a period of enzyme incubation time. The possible cause of these results might be due to the 5′-GMP was unstable with long run under enzyme hydrolysis condition. The 5′-GMP was destoryed by the enzyme called 5′-nucleotidase, degrades 5′-nucleotides into 5′-nucleosides and their corresponding bases [63,68]. This enzyme may be contained in crude extract from malt rootlets.

To prove those of assumptions, the 5′-GMP stability was tested (Figure 24) and found that the 5′-GMP was rarely destroyed. This result indicated that the enzyme incubation condition (initial pH 6.2-6.4, 60°C for 20 hr) was rarely effect on 5′-GMP stability. The reason of this result was supported by those reviews of Nagodawithna, 1995 [42] which stated that 5′-GMP are sensitive to pH values below 3 especially when the temperature is higher than 100°C. Under these conditions, the ribose linkage of the 5′-nucleotide has shown greater susceptibility compared to the phosphomonoester linkage resulting in a rapid release of the purine base. However, at higher pH levels, perhaps close to neutrality, 5′-nucleotides remained more stable even at higher temperatures. Furthermore, no appreciable destruction of 5′-nucleotides in the vicinity of the neutral pH was noted even under deep-frying conditions (180°C for 2-3 min).

For the sample with non-pretreated crude extract (control), gave the highest relative decreasing of 5′-GMP (16%). On the other hand, sample with pre-treated crude enzyme (100°C for 10-30 min or 90°C for 5 min) the 5′-GMP contents were slightly decreased. These results seem to confirm the hypothesis that the 5′-

nucleotidase exactly presented in crude malt rootlet extract and able to destroy the 5′-nucleotides in yeast extract. This result is supported by a report of Bowles LK, 1991 [70] who described that the 5′-nucleotidase can be found in rapidly proliferating parts of germinating seeds, such as the stems and rootlets of seeds which can be malted, such as oats, barley, wheat, corn and rice. This makes the crude extracts from these materials unsuitable for use in preparing 5′-nucleotides from RNA. Kirchhoff H *et al* 1970, [73] also disclosed that the crude enzyme extracted from seeds, germs, sprouts and parts thereof which are derived from di or mono-cytoledones, such as rice, barley including the sprouts accruing in the production of malt was still present enzyme 5′-nucleotidase, an enzyme which would split phosphoric acid from the 5′nucleotides, although purified by precipitation with acetone or alcohol.

As the result of this experiment, pre-treatment concentrated crude extract by heating at 72°C for 5 min was chosen as a suitable condition for inactivation the 5′-nucleotidase. However, the reduction of 5′-GMP content was still presented (92.85 % remaining 5′-GMP).

The further determination of appropriate condition for 5′-nucleotidase inactivation was studied by ranging the heating temperatures form 60-80°C and heating times from 2-180 min. The results (Figure 26 and Figure 27) can be concluded that the optimum condition was heating at 70°C for 7 min or 65°C for 30 min. This finding is in good agreement with those reported by Kirchhoff H *et al*, 1970 [73] who described that the crude extract from sprouts, germs including the sprouts from malt industry was used as an aqueous extract containing a 5′-phosphodiesterase for 5′-nucleotides production. These aqueous extracts were heated prior to use for a short period of time, at a temperature between 55-65°C for 15 to 25 min. Laufer L *et al*,

1976 [71] who also described that the enzymatic medium (extract from proliferating parts of germinating seeds such as wheat malt, rice malt, rye malt or bean shoots, which are normally dry) was heated at a temperature of 70-75 °C for about 5 min with agitation entire period before used as a source of enzyme. It is believed that heat treatment destroyed the 5′-nucleotidase. Tandkawa T *et al*, 1981 [4] also described that the enzyme solution obtained by extracting broken malt roots with water and then heating the clear filtrate at a temperature of 60-65°C for 5 to 10 min was used as crude enzyme for production process of yeast extract containing flavoring. Bowles LK, 1988 [70] also reported about a process for producing a 5′-phosphodiesterase enzyme preparation with low 5′-nucleotidase content by heating concentrated enzyme extract from ground barley malt sprouts at a temperature of between 60°C and 65°C for about 15 to 60 min. The enzyme preparation obtained had a ratio of 5′-phosphodiesterase unit to 5′-nucleotidase unit of at least 7:1 which is satisfiable ratio for producing a 5′-phosphodiesterase enzyme preparation.

However, both enzyme pre-treatment conditions, 70°C for 7 min and 65°C for 30 min, the relative decreasing of 5′-GMP was still present (8 - 9%). This data was not quite different from the data of previous pre-treatment study (72°C for 5 min). Therefore; although the pre-treatment of concentrated crude extract at optimum condition, the 5′-nucleotidase activity still was not completely inhibited because the pre-treatment conditions have to be carried out on the basic of compromise between remaining 5′-phosphodiesterase activity and 5′-nucleotidase inactivation.

6. Effect of phosphodiesterase concentration on 5′-GMP level

Results presented in Figure 21 suggest that the level of phosphodiesterase may not have been optimal to achieve a maximal level of 5′-GMP. Indeed, it was found that a maximal level of 5′-GMP (0.93% w/v, Figure 29) could be obtained by adding at least 160 unit pre-heated phosphodiesterase 100 mL of yeast extract. It should be noted that the reaction conditions as used in the assay of phosphodiesterase activity, i.e in a buffer only, differ from yeast extract : for instance, it has been shown that cysteine concentration as low as 0.1 mM reduce enzyme activity by 50 % (Ai-Yu *et at.* 1993). This is relevent as yeast extract contains considerable amounts of free amino acids. Hence it can be expected that *in situ* activity of the enzyme will be considerably lower than expected on the basis of the assayed activity.

As compared to commercial high nucleotide extracts which contain up to 2 % (w/w) 5′-GMP (Gist-Brocades, The Netherlands and Ohly, Germany, product information sheet; Peppler 1982) the maximal level reached in the present study (ca 0.9 %w/w) with brewer's yeast is approximately 50 % lower. It should be noted that according to manufacturers claims, the former extracts are made from specially selected "high RNA" (baker's) yeasts. In a standard autolysed baker's yeast extract, guanosine and 5′-GMP made up a total of ca 1.1 % of the dry weight, with guanosine accounting for more than 80 % of this; Peppler 1982. Furthermore, it is well know that RNA-levels in yeast are closely correlated to specific growth rate [89]. Brewer's yeast (*S. uvarum*, formerly *carslbergensis*) grows very slowly under the low temperature conditions employed in the production of lager beer and will thus have a relatively low RNA-content.

7. Cost estimation of 5′-GMP-rich yeast extracts

The cost estimate as shown in Table 7 found that more than a half of operating cost derived from a step of crude rootlet extract precipitation. For exsample, a cost of ammonium sulfate was 51.6% of total cost and a cost of process water for salt removing was 16.2% of a total cost. As this estimating, although a crude concentration able decreases a total volume for operating but it was also extremely increases the cost of production. For a large volume of production, this step may be therefore improved such as using a membrane filtration instance of precipitation.

8. Determination of RNA content in samples of preparation of 5′-GMP-rich yeast extracts

Determination of RNA content of samples in the preparation process of 5′-GMP-rich yeast extracts was divided into 3 sections. Raw material pre-treatment section (Table 7), the RNA contents of debittered and non-debittered yeast cells were not statistically significant different ($P > 0.05$). These results indicated that debittering process by alkaline wash was slightly effect to the RNA content of yeast cells. The reason of this result was corresponding to the reason of previous studied in that debittering brewer's yeast cell by alkaline wash was carried out by removing the bitterness substance eliminate hydrogen bonds and other weak forces of bitterness substance from the surface of yeast cell wall [19,20]. So, debittering process should not effect to the RNA content of yeast cells.

For RNA extraction section, it was found that the RNA content of yeast extract and cell debris of both before and after RNA extraction were similar (Table 7). The reason might be due to most of intracellular RNA was already extracted from the

yeast cell during autolysis, so that after RNA extraction the RNA content was merely increased in yeast extract. As these results show that the RNA extraction seem to be slightly advantage for the preparation process of 5′-nucleotide-rich yeast extracts. This fact should be considered the cost if it was done in large-scale production.

Noticeably, it was found that the RNA content of both debittered and non-debittered cell debris were contained nearly a half of RNA content of yeast extract. The one of benefit way to improve the production process was choosing the appropriate procedure to transfer the RNA content from cell debris into yeast extract.

Considering the RNA content and 5′-GMP content of yeast extract at various time of enzyme hydrolysis (Table 8), it was found that the RNA content was sharply decreased at the first 2 hr of incubation and then somewhat constant throughout the run. For 5′-GMP content, it was markedly increased during the first 4 hr of enzyme hydrolysis and then gradually decreased after 8 hr of incubation.

9. Study of 5′-GMP produced from pure RNA

The objective of this experiment was studied a efficiency of 5′-GMP forming from various concentration of pure RNA using enzyme extracted from dried malt rootlets. From the result (Figure 30), it can be concluded that a forming of 5′-GMP content was correlated to RNA concentration. Increasing of RNA concentration cause increasing of 5′-GMP content. Figure 31 shows the relationship between the concentration of starting substrate pure RNA and initial velocity of enzyme.

The reason of this result might be described by the basic concept of enzyme kinetic in that the relationship between initial velocity and substrate concentration was hyperbola. At low level of substrate, increasing of substrate concentration cause

sharply increased the initial velocity. After that, the initial velocity of enzyme was gradually slow with increasing of substrate concentration. Finally the initial velocity was level off at one level of substrate concentration [8]. From this reason it can be concluded that the initial velocity trend to increase with increasing of RNA concentration.

Considering the RNA conversion to 5′-GMP (%) (Figure 32) the percent conversion of 1.0 % (w/v) RNA concentration was not different from 1.5 % (w/v) RNA concentration. This result indicated that an increasing of RNA concentration from 1.0 to 1.5 % (w/v) could not promote an increasing of 5′-GMP forming. The reason of this result might be due to the reason as previously mentioned and might be due to an insufficiency of 5′-phosphodiesterase concentration, so that although an excess of the RNA, the reaction could not achieved.

CHAPTER VI

CONCLUSION

The objective of this study was to prepare 5′-GMP-rich yeast extracts by using a source of local raw material. The first part involved with determination of optimum condition of HPLC for 5′-nucleotide analysis. The chromatographic conditions studied were gradient and isocratic systems. They were varied with various factors. It was found that the optimum condition was isocratic system. The eluent was 0.05 M KH_2PO_4, pH 4.50-5.00. The flow rate was maintained at 1.5 mL/min throughout the procedure at ambient temperature. The column was μ Bondapak C_{18} and detector wavelength was 254 nm.

The second part involved with the preparation process. Spent brewer's yeast from brewery industry was used as a source of yeast extract and dried malt rootlets from malting industry was used as a source of enzyme.

The first section of the preparation process was the determination of suitable type of rootlets for use as a source of 5′- phosphodiesterase. Barley and rice grains were cultivated in a laboratory. At the 3^{rd} – 5^{th} day of malt rootlets germination and the 7^{th} – 9^{th} day of rice rootlets germination, the enzyme activity of rootlets was assayed. The results show no different enzyme activity of these rootlets. This investigation shows that the germination time was not effect on the amount of enzyme activity. The highest specific activity was obtained from fresh malt rootlets (0.095 unit/mg protein) followed by dried malt rootlets (0.066 unit/mg protein) and rice rootlets (0.04 unit/mg protein). Dried malt rootlets was chosen as a source of enzyme by the reason of saving

cost and time. The crude rootlet extracted from dried malt rootlets was concentrated by precipitation at 40-80% saturation of ammonium sulfate. It can increase the specific activity of crude extract by 3.2 times.

The second section of the preparation was determination of optimum conditions for pre-treatment concentrated crude extract. Before concentrated crude extract was used, it was pre-treated by various methods in order to inactivate the 5′-nucleotidase. This enzyme able to degrade the 5′-nucleotides to their correspond smaller subunits such as 5′-nucleosides and bases. These subunits have no effect on flavor in food system. The pre-treatment methods studied were adding with inhibitor ($Zn.Ac_2.H_2O$), heating at various times (2 - 180 min) and temperatures (60 - 100°C). This study found that the optimum condition was heating the concentrated crude extract at 70°C for 7 min or 65°C for 30 min. However, the reduction of 5′-GMP content was still present (92.85 % remaining 5′-GMP).

The third section was determination of optimum condition for preparation of 5′-GMP-rich in yeast extract. The studied factors were autolysis time, enzyme hydrolysis time and enzyme concentration. This preparation process was two-step process; autolysis and enzyme hydrolysis.

For autolysis, yeast slurry was prepared at 15 % solids content, initial pH 5, incubated at 50°C for 0 - 24 hr. After autolysis, yeast suspension was extracted for RNA by adjusting the suspension to pH 6.2-6.4 and incubated at 95°C for 2 hr. This extraction was expected to extract remaining RNA in yeast cell to surrounding media. From the determination of RNA content of yeast extract both before and after RNA extraction, it was found that they were not statistically significant different ($P > 0.05$). This means that RNA extraction seems to be useless for on the preparation process.

For enzyme hydrolysis step, yeast extract was mixed with pre-treated concentrated crude extract at a concentration of 0-300 units per 100 mL of yeast extract (5% solids content). The mixture was adjusted to pH 6.2-6.4 and incubated at 60°C for 0 - 44 hr.

This study found that the optimum autolysis time was 8-12 hr and the optimum enzyme hydrolysis time was 8-14 hr. Longer autolysis and enzyme incubation periods caused a decrease in the 5′-GMP content. The optimum enzyme concentration was 160 units/100mL yeast extract. Enzyme concentration more than this level showed no effect on the 5′-GMP content. This optimum condition gave the highest 5′-GMP content of, 0.93 % (w/w) of yeast extract. This study was also found that debittering brewer's yeast by alkaline wash (use 2% sodium carbonate, adjust to pH 10) slightly decreased the 5′-GMP content of yeast extract roundly 0.1 % (w/w).

The cost estimation of 5′-GMP-rich yeast extract produce for 500 kg batch was approximately 423 Baht/kg. The major cost of process came from a step of crude malt rootlet extract precipitation.

The last section involve with investigation of conversion efficiency of 5′-GMP from various starting substrate RNA concentrations. The results show that the initial RNA concentration of 1.0 % (w/v) gave the 5′-GMP content one time higher than those of 0.5 % concentration where as the RNA concentration of 1.5 % (w/v) gave similar result as those of 1.0 %. The result might be concluded that initial RNA concentration of more than 1.0 % (w/v) was not effect on the conversion efficiency.

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APPENDIX A

Determination of protein content by Lowry method [82]

Principal and application

The Lowry method is based on a reduction of the Folin-Ciocalteau reagent, composed of a mixture of phosphomolybdic and phosphotungstic acid, by oxidation of tyrosine, tryptophan, and, to a lesser extent, cystine, cysteine, and histidine residues on the polypeptide side chains. The oxidation-reduction reaction is accompanied by the formation of a characteristic blue color [90].

Chemicals

1. Reagent A - 1% (w/v) copper sulphate solution
2. Reagent B - 2% (w/v) Sodium-potassium tartrat ($C_4H_4KNaO_6.4H_2O$) solution
3. Reagent C - 0.2 M Sodium hydroxide (NaOH) solution
4. Reagent D - 4% (w/v) Sodium carbonate (Na_2CO_3) solution
5. Reagent E - The mixture reagent of 49 mL of reagent C and 49 mL of reagent D follows by adding 1 mL of reagent A and 1 mL of reagent B. The mixture reagent freshly prepare before each determination.
6. Reagent F - The mixture of Folin-Ciocalteau reagent and determineralized water at ratio 1:1.
7. Bovine serum albumin (BSA) 50, 100, 150, 250 and 350 µg/mL as a standard.

Procedure

1. Pipette exactly 0.5 mL of sample solution into glass test tube.
2. Add 2.5 mL of reagent E.
3. Mix the solution well and incubate at room temperature for 10 min in the dark.
4. Add 0.25 mL of reagent F into that test tube. Mix the solution well and incubate again at room temperature for 30 min in the dark.
5. Measure absorbance of solution mixture at 750 nm using sample buffer as a blank solution.

Calculation

$$\frac{OD_{750}}{Slope} \times Dilution = Protein\ content\ (mg)$$

OD_{750} = Optical density at 750 nm

Slope = Slope of calibration curve

Dilution = Dilution value of sample

APPENDIX B

Initial concentration of ammonium sulphate	*Final concentration of ammonium sulphate. % saturation at 0°C*																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	g solid ammonium sulphate to add to 100 ml of solution																
0	10.7	13.6	16.6	19.7	22.9	26.2	29.5	33.1	36.6	40.4	44.2	48.3	52.3	56.7	61.1	65.9	70.7
5	8.0	10.9	13.9	16.8	20.0	23.2	26.6	30.0	33.6	37.3	41.1	45.0	49.1	53.3	57.8	62.4	67.1
10	5.4	8.2	11.1	14.1	17.1	20.3	23.6	27.0	30.5	34.2	37.9	41.8	45.8	50.0	54.5	58.9	63.6
15	2.6	5.5	8.3	11.3	14.3	17.4	20.7	24.0	27.5	31.0	34.8	38.6	42.6	46.6	51.0	55.5	60.0
20	0	2.7	5.6	8.4	11.5	14.5	17.7	21.0	24.4	28.0	31.6	35.4	39.2	43.3	47.6	51.9	56.5
25		0	2.7	5.7	8.5	11.7	14.8	18.2	21.4	24.8	28.4	32.1	36.0	40.1	44.2	48.5	52.9
30			0	2.8	5.7	8.7	11.9	15.0	18.4	21.7	25.3	28.9	32.8	36.7	40.8	45.1	49.5
35				0	2.8	5.8	8.8	12.0	15.3	18.7	22.1	25.8	29.5	33.4	37.4	41.6	45.9
40					0	2.9	5.9	9.0	12.2	15.5	19.0	22.5	26.2	30.0	34.0	38.1	42.4
45						0	2.9	6.0	9.1	12.5	15.8	19.3	22.9	26.7	30.6	34.7	38.8
50							0	3.0	6.1	9.3	12.7	16.1	19.7	23.3	27.2	31.2	35.3
55								0	3.0	6.2	9.4	12.9	16.3	20.0	23.8	27.7	31.7
60									0	3.1	6.3	9.6	13.1	16.6	20.4	24.2	28.3
65										0	3.1	6.4	9.8	13.4	17.0	20.8	24.7
70											0	3.2	6.6	10.0	13.6	17.3	21.2
75												0	3.2	6.7	10.2	13.9	17.6
80													0	3.3	6.8	10.4	14.1
85														0	3.4	6.9	10.6
90															0	3.4	7.1
95																0	3.5
100																	0

Table 11 The amount of solid ammonium sulfate to be added to a solution to give the desired final saturation at 0 °C

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