AN ABSTRACT OF THE THESIS OF

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in FOOD SCIENCE (Major) presented on March 8, 1968 (Date)

Title: IDENTIFICATION OF SOME ESTERASES OF THE GREEN BEAN (PHASEOLUS VULGARIS L.)

Abstract approved: Dr. Morris W. Montgomery

This investigation was conducted to differentiate the esterases in a water extract of freeze-dried green beans on the basis of inhibitor and substrate specificities. An attempt was made to classify these esterases according to the criteria used for animal esterases.

Activity of the esterases was measured manometrically using the Gilson differential respirometer. Aqueous extracts of green beans were found to hydrolyze the acetyl, propionyl, and n-butyryl esters of glycerol, phenol, and 2-naphthol-6-SO_3Na. No hydrolysis of triolein or long-chain 2-naphthol-6-SO_3Na esters was noted, indicating the absence of lipases. A small amount of activity was observed when the choline esters served as substrates, but this was attributed to esterases other than cholinesterases. Optimum activity of the extract on triacetin, tripropion, tributyrin, phenyl acetate, and phenyl propionate occurred at pH 7.2.

The effects of organophosphorus inhibitors, diethyl p-nitrophenyl thiophosphate, tetraethyl pyrophosphate, and diisopropylphosphorofluoridate, at concentrations ranging from 10^{-1} M to 10^{-10} M on the esterase activity was studied. These data show that the green bean extract contains at least three esterases. One was resistant to certain organophosphorus compounds, suggesting similarity to animal arylesterases (aryl ester hydrolase, EC 3.1.1.2). Various concentrations of organophosphorus compounds inhibited the activity of the two other esterases. These were classified as carboxylesterases (carboxylic ester hydrolase, EC 3.1.1.1).

Identification of Some Esterases of the Green Bean (*Phaseolus vulgaris* L.)

by

Teryl Beebe Putnam

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1968

APPROVED:

Assistant Professor of Food Science and Technology
in charge of major

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Date thesis is presented March 8, 1968

Typed by Donna Olson for Teryl Beebe Putnam

ACKNOWLEDGEMENTS

Sincerest appreciation is extended to Dr. Morris W. Montgomery, Assistant Professor of Food Science and Technology, Oregon State University, for his guidance and assistance throughout the course of this investigation and during the preparation of this manuscript. Thanks are due to Dr. Allen F. Anglemier, Associate Professor of Food Science and Technology, Oregon State University, for his assistance in reviewing this manuscript.

This investigation was supported in part by U. S. Public Health Service Grant No. AM 09306.

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IDENTIFICATION OF SOME ESTERASES OF THE GREEN BEAN (PHASEOLUS VULGARIS L.)

INTRODUCTION

Esterases are enzymes which catalyze the hydrolysis of ester linkages. They are generally differentiated from lipases on the basis of substrate solubility and the carbon chain-length of the substrates which they hydrolyze. Little is known about the physiological function of the esterases, although acetylcholinesterases, found in various animal tissues, are known to play a role in transmission of nerve impulses. It is hoped that differentiation of the esterases in various organisms will lead to some insight of their function.

In the food industry the possibilities of esterases being involved in various metabolic systems which affect flavor, texture and color of food substances are of interest. If the esterases are involved in various metabolic systems, the use of insecticides, such as certain organophosphorus (OP) compounds, which inhibit the activity of some esterases, would be of concern. These compounds could upset the metabolism of the organism and possibly affects its flavor, texture and color.

Detailed studies on animal esterases have shown that several different esterases exist. A classification for these esterases has been established, but exceptions have been found. When the functions

of the esterases are understood, a more logical classification may be devised.

Esterases also exist in many plants, however, few detailed studies have been reported. The work of Jooste and Moreland (1963), Schwartz et al. (1964), Norgaard (1968), and Carino (1968) revealed that complex esterase systems exist in plants as well as in animals.

This investigation was made to differentiate the esterases of green beans, and to attempt to classify them according to the system used for animal esterases. Since green beans are a crop of major economic importance to the State of Oregon, they were selected for this study.

REVIEW OF LITERATURE

The distinction between esterases and other hydrolytic enzymes is vague in many instances. Certain proteolytic enzymes were reported to hydrolyze several esters by the same mechanism as esterases and were inhibited by some of the most potent esterase inhibitors (Myers, 1960). Similarly certain esterases have been shown to hydrolyze amide derivatives (Myers, et al., 1957) and acid anhydrides (Wilson, 1954). This report will be concerned only with those enzymes which hydrolyze carboxylic esters.

Classification of Esterases by Substrate Specificity

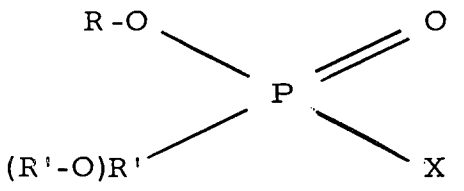
Ill-defined terms of aliesterases, simple esterases, and lipases have been used to designate the various esterases. However, overlapping specificities and indiscriminate use of these terms have caused confusion (Hofstee, 1960). Lipases, for example, were generally considered to be esterases catalyzing the hydrolysis of long-chain fatty acid esters, especially fats. Hydrolysis of short-chain aliphatic esters was catalyzed by aliesterases or simple esterases (Hofstee, 1960). These definitions changed, however, when the following observations were made. An esterase in liver was found which attacked solutions of long-chain aliphatic and aromatic esters at a much higher rate than short-chain esters, but did not

catalyze the hydrolysis of true fats (Sarda and Desnuelle, 1958). Sarda and Desnuelle (1958) also found activity of purified pancreatic lipase to be significantly pronounced only when the saturation point of the substrate was reached. These results lead to the conclusion that fatty acid esterases could be divided into those active on substrates in solution (esterases proper) and those active on insoluble substrates (lipases). Considering the characteristics of liver esterase, Hofstee (1960) suggested that "lipase" refer to fat-splitting enzymes only.

Cholinesterases refer to esterases which hydrolyze mainly choline esters. A few of these esterases were able to hydrolyze various aliphatic (Augustinsson, 1958, 1959) and aromatic (Mounter and Whittaker, 1953) esters, but they could easily be distinguished from the "esterases proper" by their extreme sensitivity to physostigmine (Richter and Croft, 1942; Myers and Mendel, 1949).

Classification of Esterases by Selective Inhibition

One of the best means of distinguishing various esterases has been the use of compounds which have an inhibitory effect on the action of some of the esterases. OP compounds, producing an irreversible inhibition, are commonly used. These substances have the general formula



where R and R' are alkyl groups and X is -F, -CN, or $-OC_6H_4NO_2$. OP compounds are interesting in that their action is confined to enzymes which hydrolyze esters. Dixon and Webb (1964) explain the action of OP compounds on esterases by the following reactions:

1. $R\text{-}C(=O)\text{-}O\text{-}R' + EH = R\text{-}C(=O)\text{-}E + HO\text{-}R'$

2. $R\text{-}O\text{-}P(=O)(R)(X) + EH = R\text{-}O\text{-}P(=O)(R')(E) + HX$

3. $R\text{-}C(=O)\text{-}E + HOH = R\text{-}C(=O)\text{-}OH + EH$

4. $R\text{-}O\text{-}P(=O)(R)(E) + HOH = R\text{-}O\text{-}P(=O)(R)(OH) + EH$

The enzyme forms the inhibitory group in a process analogous to the first stage of hydrolysis of the substrate. The first stage in the hydrolysis of the substrate was the transfer of the acyl group to the

enzyme, forming an acylated enzyme and liberating a free alcohol, as shown in reaction 1. Reaction 2 shows the first stage of the inhibition reaction involving the transfer of the phosphoryl group to the enzyme, yielding a phosphorylated enzyme and liberating HX. The second stage in the hydrolysis of the substrate was the reaction of the acylated enzyme with an acyl acceptor. This acceptor was usually water, but an alcohol or hydroxylamine could also react (Myers, 1960). Reaction 3 shows the acylated enzyme reacting with water (the acyl acceptor) to yield the free enzyme and the appropriate acid. The phosphorylated enzyme, however, was comparatively stable and hydrolyzed at a negligible rate. In the case of the less effective OP inhibitors, the phosphorylated enzyme did hydrolyze at a significant rate releasing the free enzyme with full activity (reaction 4). The interaction of the OP compounds with the esterase occurred when the phosphoryl group was attached to the hydroxyl group of one of the serine residues. Janzs and co-workers (1959) found this reactive serine to be attached in the sequence glutamic acid-serine-alanine in carboxylesterase from horse liver and in cholinesterase from horse serum. Similar sequences have been found in esterases and proteinases from various sources. The phosphoryl group was always attached to the serine residue (Oosterbaan and Cohen, 1964).

The inhibitory action of physostigmine was similar to that of

the OP compounds (Wilson, 1960). The carbamoyl or substituted carbamoyl groups were attached to the active center of the esterase.

Another compound used in differentiating esterases, p-chloromercuribenzoate (PCMB), has been shown to react with enzymes containing thiol groups to form mercaptides. However, in high concentrations, inhibition of enzymes have occurred by reactions not involving -SH groups (Sohler et al., 1952). In contrast to inhibition by OP compounds, PCMB inhibition was reversible (Dixon and Webb, 1964).

The majority of information on classification of esterases refers to work on esterases from animal origin. Some of the observations leading to the development of a tentative classification system are presented.

Although several workers (Bloch, 1943; Hottinger and Bloch, 1943) have shown the activity of some lipases and esterases from various sources was inhibited by OP compounds, Aldridge (1953a) was the first to distinguish types of esterases by the use of selective inhibitors. Working with rat and rabbit sera, he found two distinct esterases which were designated as A- and B- esterases. The esterase in rabbit serum, which hydrolyzed p-nitrophenylacetate at a higher rate than the butyrate ester, was not inhibited by diethyl-p-nitrophenyl phosphate (paraoxone or E-600) at concentrations up to 10^{-3} M. This esterase, A-esterase, was also resistant to

diisopropyl phosphoflurodate (DFP) and tetraethyl pyrophosphate (TEPP). B-esterase, found in the rat serum, hydrolyzed p-nitrophenyl butyrate faster, or as fast as, p-nitrophenyl acetate. This esterase was inhibited by paraoxone at concentrations as low as 10^{-8} M and by 10^{-7} to 10^{-8} M DFP and TEPP.

Later, Aldridge (1953b) reported that A-esterase hydrolyzed paraoxone. Myers (1960) also reported that A-esterase was capable of hydrolyzing DFP. Neither A- nor B-esterases were inhibited by eserine (physostigmine) indicating they were not cholinesterases (Aldridge, 1953a).

In subsequent work, Aldridge (1954) emphasized the importance of using a variety of substrates and inhibitors for differentiating various esterases. He found that although A-esterase of rabbit serum hydrolyzed paraoxone, A-esterase from rat pancreas showed no hydrolysis with a variety of OP inhibitors. A-esterase from rat pancreas hydrolyzed tributyrin like the A-esterase of the rabbit serum, but failed to hydrolyze the phenyl esters. When this pancreatic A-esterase was found to hydrolyze the higher glycerides, it was designated a pancreatic lipase. Myers (1956) also points out the importance of using several inhibitors and substrates when trying to differentiate esterases.

Using a series of aliphatic, aromatic, and choline esters as substrates and a variety of inhibitors, Augustinsson (1958, 1959)

proposed a classification of esterases similar to that suggested by Aldridge. Working with the blood plasma esterases from a variety of mammalian species, which he had separated by electrophoresis on cellulose columns, Augustinsson (1958, 1959) suggested the following classification of esterases:

Ayrlesterases (A-esterases): esterases which hydrolyzed phenylacetate approximately 100 times faster than phenyl butyrate. Aliphatic and choline esters were not hydrolyzed. These aromatic esterases were resistant to certain OP compounds and to physostigmine, but were sensitive to PCMB.

Aliesterases (B-esterases): esterases which hydrolyzed aliphatic and aromatic but not choline esters. These esterases were sensitive to 10^{-5} M of many of the OP inhibitors, but were not inhibited by 10^{-5} M physostigmine.

Cholinesterases (Ch-esterases): esterases which hydrolyzed choline esters at a higher rate than aliphatic or aromatic esters. They were completely inhibited by 10^{-5} M physostigmine, and were sensitive to 10^{-6} M or less concentrations of many OP inhibitors.

In addition to A- and B-esterases, Bergmann and co-workers (1957) reported a C-esterase. This esterase was not inhibited by DFP. Unlike A-esterase, however, C-esterase was activated by concentrations of PCMB which inhibited A-esterase. At higher concentrations, PCMB inhibited C-esterase.

Based on inhibitor and substrate specificities, the esterases can be differentiated and possibly be classified as A-, B-, or C-esterases. The cholinesterases can be detected by physostigmine. It is possible to recognize these groups of esterases even in crude systems using this type of study (Hofstee, 1960). However, esterases with overlapping characteristics will probably be found.

Classification of Esterases by the Enzyme Commission

In 1961 the Commission of Enzymes of the International Union of Biochemistry (1961) set up a system of classifying the enzymes based on the nature of the overall reaction catalyzed. The enzymes were divided into six main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Each class was further divided into subclasses and sub-subclasses. Esterases were designated as a subclass of hydrolases, and carboxylic ester hydrolases were a sub-subclass of the hydrolases acting on ester bonds (Dixon and Webb, 1964). Each enzyme was given a systematic name and a trivial or "working" name. The trivial name was usually shorter and often the one in current use. The trivial name carboxylesterase (EC 3.1.1.1) was designated for aliesterase or B-esterase, while carboxylic ester hydrolase was the designated systematic name. Similarly, A-esterase was given the trivial name arylesterase (EC 3.1.1.2) and the systematic name aryl ester

hydrolase. This system is well suited for enzymes which are specific for certain substrates. Many esterases, however, have wide substrate specificities and cannot be grouped by substrate specificity alone.

Plant Esterases

Information on esterases from plants is sparse in comparison with that available on esterases from animals. Although esterases have been reported in plants, studies differentiating these esterases are few.

Lipases have been reported in wheat germ (Singer and Hofstee, 1948a; Jansen, Nutting, and Balls, 1948), citrus (Jansen, Jang, and MacDonnell, 1947; Jansen, Nutting, and Balls, 1948), cottonseed (Olcott and Fontaine, 1941), castor bean (Longenecker, 1946; Ory, Angelo, and Altschul, 1960), oats (Martin and Peers, 1953), malt (Lowy, 1945), soybean (Gorback, 1941), rice embryo (Obara and Ogasawara, 1959), and pea (Lee and Wagenknecht, 1958). Esterases have been found in wheat (Sullivan and Howe, 1933; Jooste and Moreland, 1962; Mounter and Mounter, 1962), bean leaves (Rudolf and Stahmann, 1966), peas (Frankel and Garber, 1965; Norgaard, 1968), tomato and lucerne (MacDonnel et al., 1950), germinating seeds, young leaves, and "used flowers" of 12 species of Genus Collinsia (Wennstrom and Garber, 1965), maize (Schwartz, 1960),

green beans, cabbage, potato tuber, citrus albedo and flavedo, and maize kernel (Schwartz et al., 1964), spherosomes of onion (Walek-Czanecka, 1963), spherosomes of corn and tobacco (Matile, et al., 1965), shoot tips of white spruce (Born, 1963), in root tips of broad bean, Vica faba (Benes, 1962) and in seedlings of corn, cucumber, and soybean (Jooste and Moreland, 1962).

Pectinesterase occurs in various plants (MacDonnell et al., 1950), and chloroplasts of green plants contain chlorophyllase (Krossing, 1940).

A distinction between a type of esterase proper and a pectinesterase was made by Jansen and co-workers (1947) when they found an esterase in citrus which hydrolyzed the esters of acetic acid. Pectinesterase did not hydrolyze acetic acid esters. This acetylesterase also hydrolyzed acetylcholine bromide, but eserine had no effect on its action, thus, it was not a cholinesterase. The rate of hydrolysis on various glycerides and other substrates revealed that the acetylesterase was not a lipase.

Jooste and Moreland (1963) have reported more extensive studies in an attempt to characterize esterases from wheat seeds, corn, cucumber, and soybean seedlings. Using the acetate, butyrate, caprylate, and phenoxyacetate esters of 2-naphthol, they observed the effect of physostigmine, DFP, and PCMB on the hydrolysis by the various esterase extracts. These authors also made

starch-gel electrophoretic studies on the cucumber, soybean, and wheat preparations. Up to nine bands of esterase activity by some of the plant preparations were revealed. From the inhibitor and substrate studies, several different arylesterases and carboxylesterases were proposed. Also, as indicated in an earlier paper (Jooste and Moreland, 1962), an esterase hydrolyzing 2-napthylphenoxyacetate, was found. This phenoxyesterase, which was not affected by 10^{-6} to 10^{-3} M PCMB, but was slightly stimulated (17 percent) by 10^{-3} M DFP, did not fit into the classification proposed for animal esterases.

Schwartz and co-workers (1964) made a study of esterases in green beans, cabbage, potato tuber, citrus albedo and flavedo, apple, peach, mango, carrot, tomato, egg fruit, onion, and many varieties of Cucurbitaceae. High esterase activity toward indophenyl acetate was found in green beans, cabbage, potato tuber, citrus albedo and flavedo, and fruits of many cucurbits. Starch-gel electrophoresis revealed several bands of esterase activity in the different plant species.

Norgaard (1968) studied the effects of various inhibitors on a water extract of lypholyzed peas. This extract hydrolyzed, to various degrees, the acetyl, propionyl, and butyryl esters of phenol, 2-naphthol-6-SO_3-Na, and glycerol. The effect of the inhibitors DFP, TEPP, and diethyl p-nitrophenyl thiophosphate (parathion or PARA)

at various concentrations revealed as many as six esterases, five of which appeared to be carboxylesterases. No cholinesterases were found. In similar studies, Carino (1968) reported the presence of six esterases in carrots.

These more extensive studies reveal a complex system of esterases exists in many plants as in animals. The function of the majority of these esterases is not understood. Further characterization of these systems may reveal their metabolic role.

MATERIALS AND METHODS

Preparation of Enzyme Extract

Freshly harvested green beans (Phaseolus vulgaris, L., variety Blue Lake) were obtained from Blue Lake Packers, Inc., Corvallis, Oregon. The beans were cut, freeze-dried, with the temperature of the heating plates remaining below 38°C, packed in No. 2-1/2 cans under N_2, and stored at -18°C until needed.

A sample of the beans was ground for three minutes in a Waring Blendor. This powder was mixed, 1 to 10 (w/v), with cold distilled water. The slurry was placed in polyethylene centrifuge tubes, and centrifuged at 15,000 rpm (27,000 x G) for 20 to 25 minutes in a Super-Speed Servall Centrifuge located in a 4°C cold room. The supernatant was pipetted off, and a portion, to be used as a blank, was heated in a boiling water bath for 10 minutes. Both the heated and unheated portions were stored in the refrigerator until needed.

Preparation of Substrates

Substrates used in the study and their concentrations are shown in Table 1. They were prepared by the following procedure. Appropriate amounts of the chemicals were weighed into 50 ml volumetric flasks. One and one-half grams of Triton X-155 (Rohm

and Haas) were added. After two mls of two and one-half percent (w/v) gum acacia were added, the flask was shaken, diluted to volume with distilled water, and mixed. These solutions, except the 2-naphthol-6-SO_3Na esters, were emulsified in a water-cooled semi-micro container of the Waring Blendor at maximum speed for two minutes. Substrate solutions were refrigerated in glass-stoppered bottles, but were brought to room temperature before use.

Triton X-100 was found to promote solubilization of some substrates, and to enhance the activity of certain esterases (Allen, Allen, and Licht, 1965). Gum acacia was reported by Mounter and Mounter (1962) to have no effect on the rate of hydrolysis of triacetin by wheat germ esterase.

Preparation of Inhibitors

OP inhibitors TEPP, DFP, and PARA were prepared by the following method using TEPP as an example. Approximately 0.8 grams of a 40 percent stock solution of TEPP was accurately weighed into a 25 ml volumetric flask and made to volume with one percent Triton X-100 and mixed. The molarity was calculated. The volume of the solution needed to make 25 ml of 4.0×10^{-2} M TEPP was calculated and placed in a 25 ml volumetric flask. The volume was made up with the one percent Triton solution. Serial dilutions of the 4.0×10^{-2} M solution were made to give solutions of

4.0×10^{-3} M through 4.0×10^{-10} M. These concentrations resulted in a final concentration in the reaction mixture of 1.0×10^{-2} M through 1.0×10^{-10} M.

Series of similar solutions were made for DFP and PARA, except that the PARA required homogenization in a Waring Blendor for 30 seconds to obtain an emulsion and the series of dilutions for PARA included 4.0×10^{-1} M.

Assay Procedure

Esterase activity was determined by manometric techniques similar to those described by Forster, Bendixen, and Montgomery (1959), and Norgaard (1968), using a Gilson differential respirometer. The H^+ from the acid produced as the substrate was hydrolyzed, shifts the following equilibrium to the right, evolving CO_2:

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 \text{ (dissolved)} \rightleftharpoons CO_2 \text{ (gas)}$$

This volume of CO_2 evolved from the bicarbonate buffer was used as the measurement of activity. A total liquid volume of four ml was used in all the studies. Main compartments of the flasks contained one and one-half ml of $NaHCO_3$ buffer; one ml of either water, one percent Triton X-100, or inhibitor; and one ml of the bean extract, either heated or unheated. The side arm compartments contained 0.4 ml of substrate and 0.1 ml of bicarbonate buffer.

Flasks were gassed for ten minutes with a mixture of five percent CO_2 and 95 percent N_2 and equilibrated for at least ten minutes, while shaking in a 37°C water bath. Before the equilibration period, the pressure on the system was adjusted to 770 mm Hg. After equilibration, the flasks were tipped at succeeding 30-second intervals and readings were taken at ten-minute intervals for 30 minutes. The activity was measured in terms of A_{30}, the µl of CO_2 evolved in 30 minutes by the sample flask minus the µl of CO_2 evolved in 30 minutes by the control flask, and as enzyme units, the microequivalents of substrate hydrolyzed per minute per ml of original enzyme extract.

Room temperature during each assay was noted, since this affected the manometers and tygon tubing in the system which were not submerged in the 37°C water bath. Corrections for these day to day temperature differences were calculated according to the formula of Gregory and Winter (1965). The proper correction factor was multiplied by the A_{30} giving the corrected A_{30} (Norgaard, 1968).

RESULTS AND DISCUSSION

In this investigation of the esterase activity of green beans, preliminary studies were made to determine the substrates hydrolyzed by the bean extract and the pH optimum of this activity. A study of inhibition by OP compounds was then made to differentiate the esterases in beans.

Substrate Study

The first step made in studying the esterase activity of green beans was to determine the substrates hydrolyzed by the extract. These studies were carried out at 37°C at atmospheric pressure, with no corrections for room temperature variation. Since preliminary studies with triacetin and a titrimetric procedure indicated an optimum pH of bean esterases to be pH 7.7, this pH was used in these substrate studies.

The activity varied widely among the different groups of substrates and within groups. Therefore, various dilutions of the bean extract were used to maintain a constant rate of hydrolysis through the incubation period. These extract dilutions, substrate concentrations, and activities are presented in Table I.

Considerable activity was obtained with acetyl, propionyl, and butyryl esters of each group of substrates. Propionyl esters

were hydrolyzed more rapidly than acetyl and butyryl esters of glycerol and phenol. Activity increased as acyl chain-length was increased through the butyryl ester of 2-naphthol-6-SO_3Na. Longer chain esters of 2-naphthol-6-SO_3Na and triolein were not hydrolyzed significantly. This suggests the absence of esterases capable of hydrolyzing soluble long-chain esters or lipases in the bean extract. Lipases were defined as specific for the long-chain, insoluble esters (Sarda and Desnuelle, 1958).

Table I. Activity of green bean extract on various substrates.

Substrate	Concentration (M)	Bean Extract Dilution (v/v)	Activity Enzyme Units
Triacetin (TA)	0.23	1 to 10	2.03
Tripropion (TP)	1.0	1 to 10	2.98
Tributyrin (TB)	1.0	1 to 10	0.93
Triolein	0.5	no dilution	0.01
Phenyl acetate (PA)	1.0	1 to 10	3.39
Phenyl propionate (PP)	1.0	1 to 10	4.91
Phenyl butyrate (PB)	1.0	1 to 10	4.02
2-naphthol-6-SO_3Na esters			
acetyl (NA)	0.06	1 to 10	0.61
propionyl (NP)	0.06	1 to 10	1.26
butyryl (NB)	0.06	1 to 10	1.34
hexyl	0.06	no dilution	0.00
octyl	0.06	no dilution	0.00
decyl	0.06	no dilution	0.00
Acetyl choline iodide	1.0	no dilution	0.17
Propionyl choline iodide	0.1	no dilution	0.05
Butyryl choline iodide	0.1	no dilution	0.09

The phenyl esters were hydrolyzed to a greater extent than the triglyceride, naphthyl and choline esters. It is interesting to note that as the polarity of the alcohol moiety of the substrates increased, the activity decreased. This is in agreement with Dixon and Webb (1964), who postulated that van der Waal forces played a major role in enzyme-substrate binding of horse liver carboxylesterase.

The choline esters were hydrolyzed only slightly. This hydrolysis was probably due to arylesterases or carboxylesterases rather than cholinesterases. Jansen and co-workers (1947) reported the hydrolysis of acetylcholine by citrus acetylesterase. Likewise, Mounter and Mounter (1962) found the choline esters were hydrolyzed by wheat germ esterase. Neither of these esterases were eserine sensitive. Hofstee (1960) mentioned that cholinesterases had not been found in plant tissue. Schwartz (1964) reported that the fruit of the Green Hubbard, the flavedo of three citrus fruits, and the orange and lemon albedo contained an acetylcholinesterase. This is questionable however, since this conclusion was based on the hydrolysis of acetylcholine bromide, which was not affected by 10^{-5} M DFP and was slightly stimulated, not inhibited, by 10^{-5} M eserine. Cholinesterases are by definition inhibited by 10^{-5} M eserine and by low concentrations of OP compounds. It is very likely that plants do not have cholinesterases, since the function of acetylcholinesterases in animal tissues is concerned with

synaptic transmission (Iwatsubo, 1965; Augustinsson 1960; Wilson, 1960).

pH Study

A pH study was conducted to determine the optimum pH of the esterases in green beans. Concentrations of sodium bicarbonate buffers in the reaction mixtures were varied to obtain the desired pH (Umbreit, Burris, and Stauffer, 1964).

First, a range of pH values including 6.0, 6.5, 7.0, 7.5, and 8.0 were used to define the general area of the pH optimum. Later a series of assays were made at pH values from 6.4 to 8.0 at 0.2 intervals. The substrates used were PA, PP, TA, TP, and TB. The results, expressed as percent of maximum activity, are shown in Figures 1 and 2. The optimum pH was approximately 7.2 for these substrates. The increased hydrolysis of triacetin at the higher pH conditions could be due to basic hydrolysis or to an enhancement of the catalytic effect of one or more esterases on triacetin by OH^- ions. Bergmann (1958) describes this effect, which occurred with the hydrolysis of p-nitrophenyl acetate by C-esterase from hog kidney.

The pH optima of various esterases from different sources are presented in Table II.

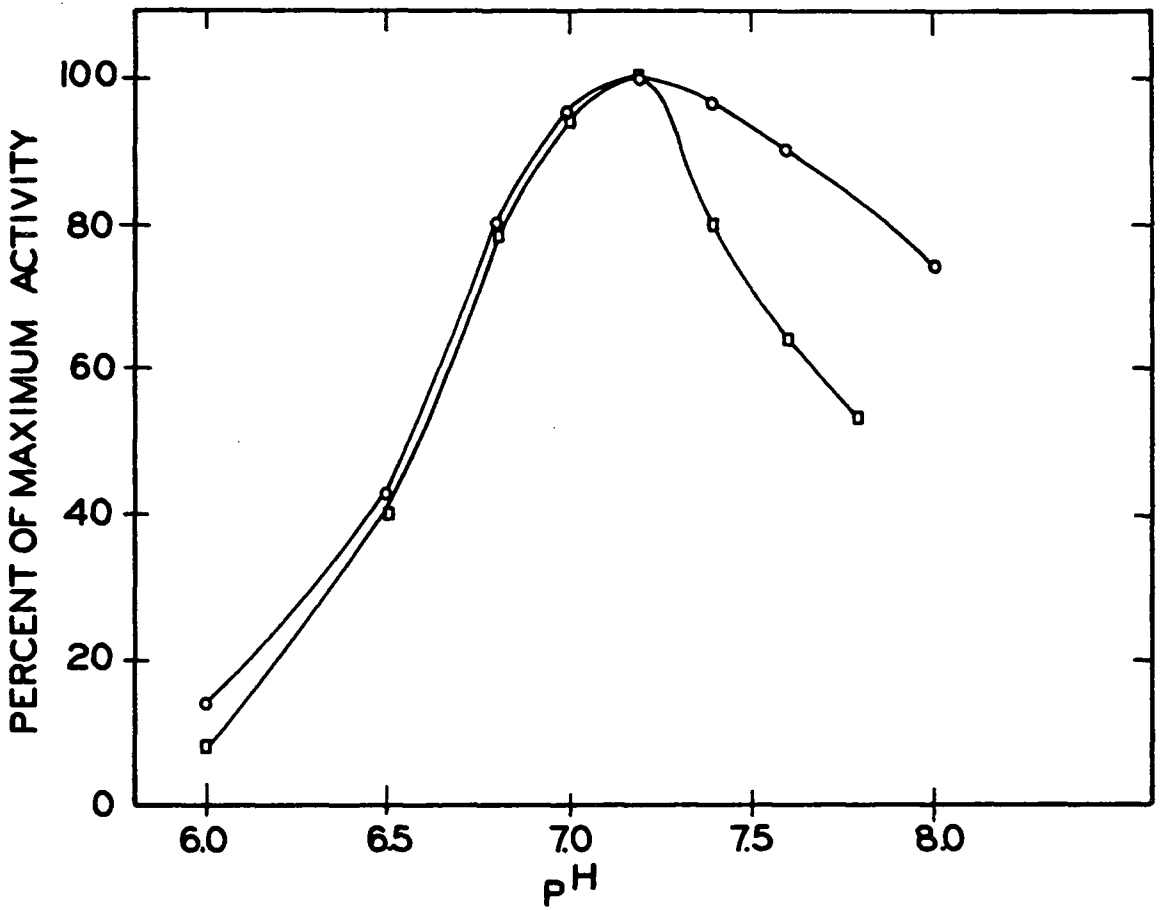


Figure 1. Effect of pH on the hydrolysis of phenyl acetate (□——□) and phenyl propionate (○——○) by green bean extract.

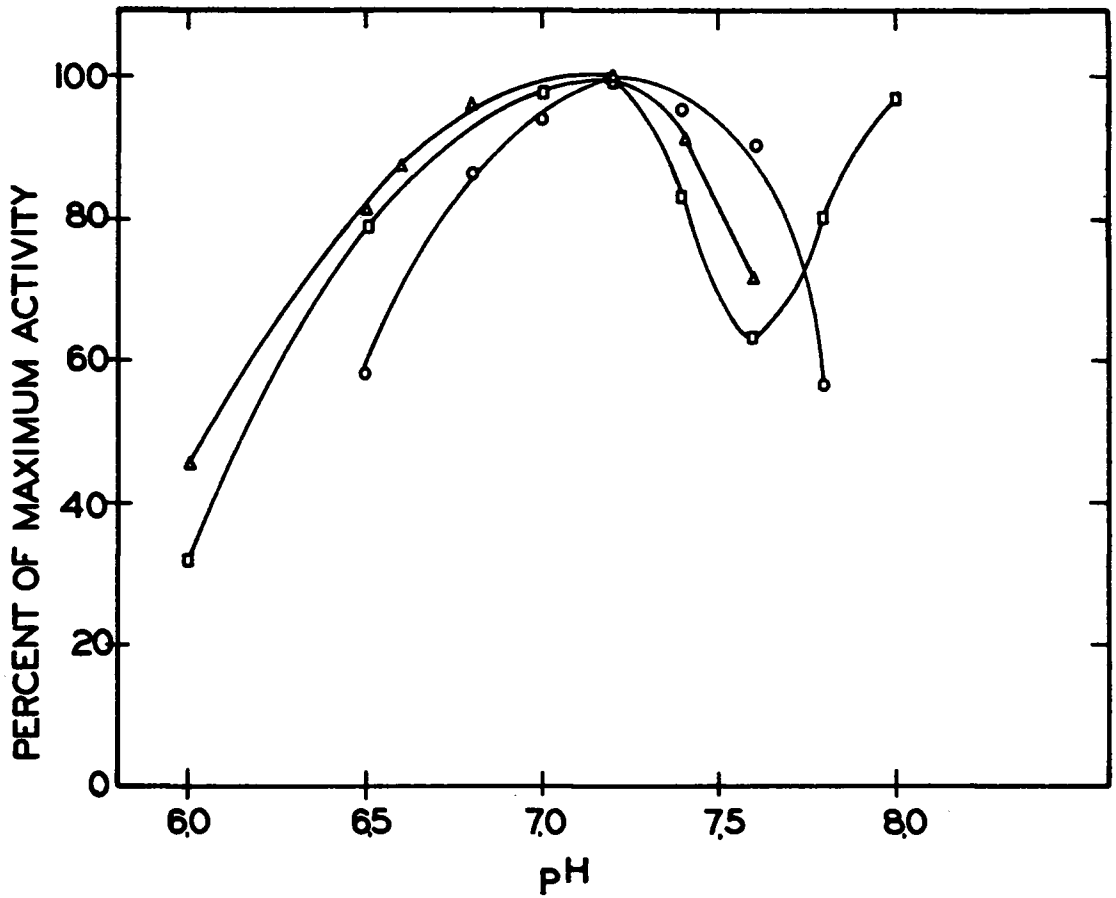


Figure 2. Effect of pH on the hydrolysis of triacetin (□——□), tripropion (○——○), and tributyrin (△——△) by aqueous extract of green bean.

Table II. Reported pH optima of esterases and lipases.

Esterase source	Optimum pH	Substrate	Investigator
Cottonseed			Olcott and
Lipase	7.0-8.0	corn oil	Fontaine (1941)
Castor Bean			
Lipase I	4.7-5.0	---	Longenecker (1946)
Lipase II	neutral to alkaline	---	
Soybean			
phenoxyesterase	6.0	2-naphthyl-phenoxyacetate	Jooste and Moreland (1962)
Hog Kidney			
C-esterase	8.0	Phenyl acetate	Bergmann (1958)
Human Plasma			
Aromatic esterase	7.5	Phenyl acetate	Mounter and Whittaker (1953)
Arylesterase I	7.8	p-nitro-phenyl	Augustinsson
Arylesterase II	9.5	phosphate	(1964)
Purified plasma			
Butyryl cholinesterase	broad opt. stable at 6.0	---	Augustinsson (1960)
Rabbit serum			
E600-esterase	7.4-7.6	Paraoxone	Aldridge (1953)

There is a wide variation of optimum pH and no definite pattern between the optimum pH for plant and animal esterases exists. Aldridge (1954) and Augustinsson (1960) state that the pH optimum of esterases varied with different substrates and between a mixture of esterases or a single esterase.

Inhibitor Studies

Since the activity of esterases is affected by various OP compounds, these compounds have been used to classify esterases (Adlridge, 1953a, b). Aldridge (1953a) distinguished two serum esterases, which hydrolyzed the same substrate, by their sensitivity to OP compounds. The one which was inhibited gave a sigmoid curve when activity was plotted against negative $\log_{10}$ of the molar inhibitor concentration (pI). A sigmoid curve indicated that one esterase was inhibited. It is possible to have two esterases with the same inhibitor sensitivity and a single sigmoid curve would result. Only when different sigmoid curves or when a double sigmoid curve is obtained can two or more esterases sensitive to OP compounds be distinguished.

When a sensitive and a resistant enzyme exist in the same preparation and hydrolyze the same substrate, the sigmoid curve will form a plateau before 100 percent inhibition is reached. This plateau is the point from which the percent activity of each esterase on the substrate can be calculated. A plateau is also formed when two esterases having different inhibitor sensitivities exist together and hydrolyze the same substrates.

One esterases is inhibited at a low concentration and a plateau is formed and continues until the concentration is reached at which

the less sensitive esterase is inhibited. Myers, Tol, and de Jonge (1957) reported a double sigmoid curve when the esterase activity of several strains of Mycobacteria was plotted against the pI of diisopropyl p-nitrophenyl phosphate.

Effects of the inhibitors PARA, TEPP, and DFP at concentrations ranging from pI 1 to 10 on the esterase activity of a water extract of green beans are shown in Figures 3, 4, and 5. Percent inhibition was calculated by subtracting the A_{30} of the inhibited sample from the A_{30} of the control sample (no inhibitor), dividing by the A_{30} of the control and multiplying by 100. The results were plotted as percent inhibition versus the pI.

In general, double sigmoid curves were obtained when pI of PARA was plotted against the percent inhibition (Figure 3). This indicates that at least two esterases were present in the bean extract which were active toward the substrates and sensitive to the OP compounds. The most sensitive esterase was inhibited between pI 8 and 5 of PARA and was responsible for 40 percent of the activity with the phenyl esters, 25 percent with TP, 20 percent with NP, 15 percent with TA, TB, and NA, and five percent with NB. The second esterase was inhibited between pI 4 and 1 of PARA. This esterase accounted for 75 to 85 percent of the activity toward the glyceryl esters, 60 percent toward the phenyl esters, NP and NB, and 45 percent toward NA. These esterases hydrolyzed all the phenyl esters

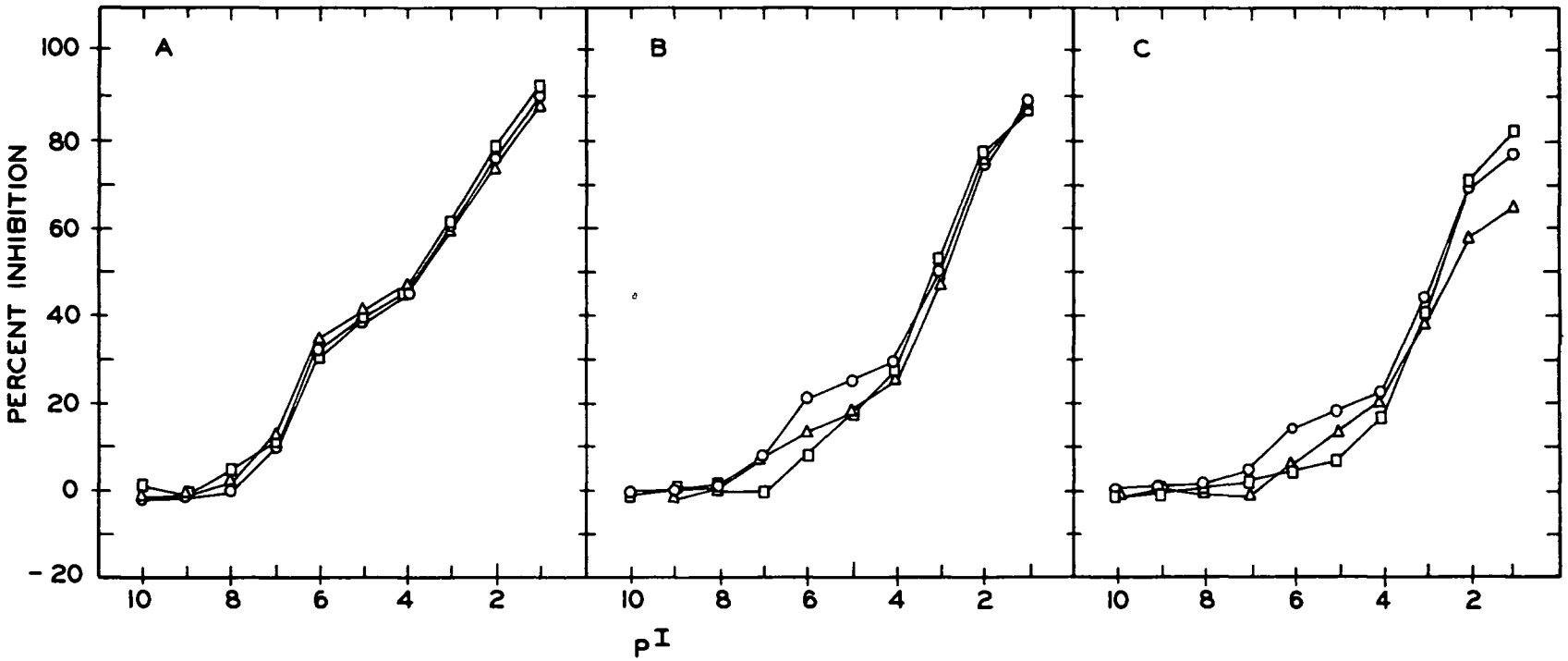


Figure 3. Inhibition by various PARA concentrations of the hydrolysis of acetyl (△——△), propionyl (○——○), and butyryl (□——□) esters of phenol (A), glycerol (B), and 2-naphthol-6-SO_3Na (C) by green bean extract. pI is the negative $\log_{10}$ of molar inhibitor concentration.

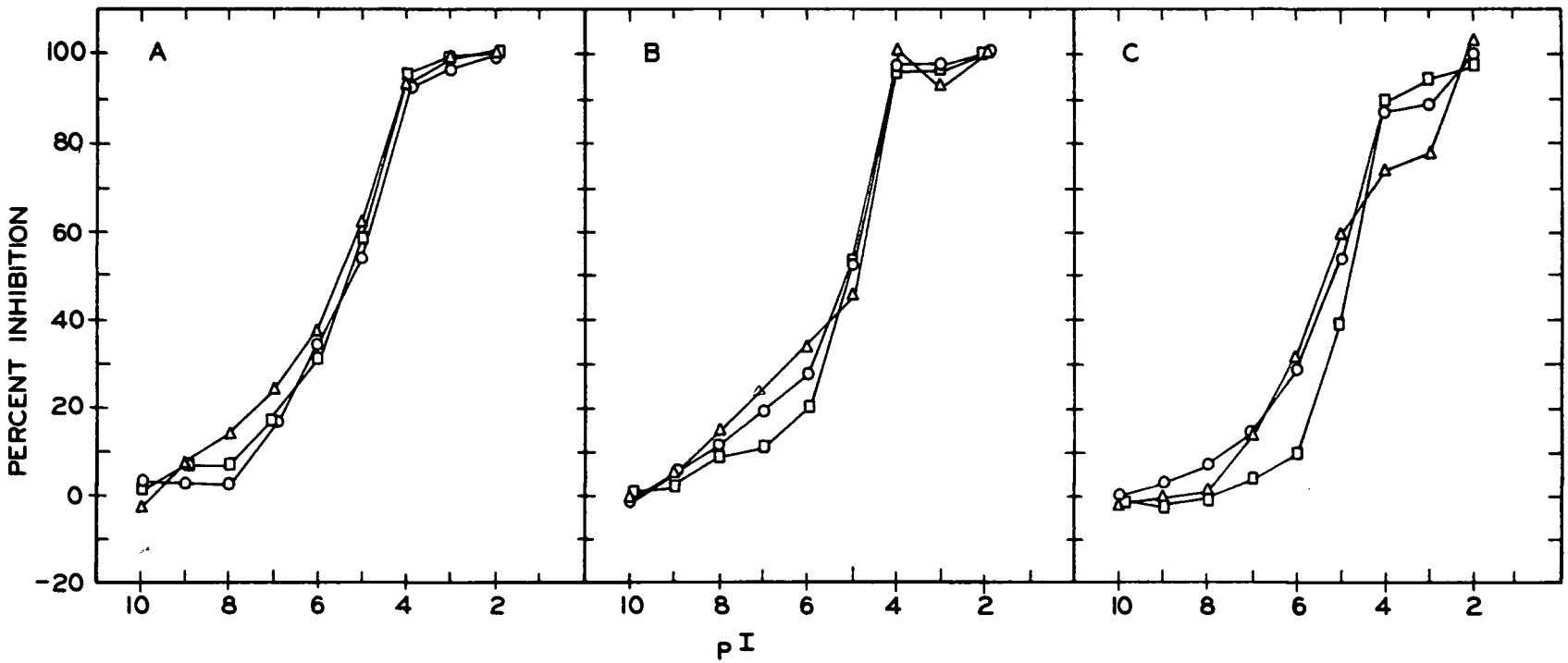


Figure 4. Inhibition by various TEPP concentrations of the hydrolysis of acetyl (△——△), propionyl (○——○), and butyryl (□——□) esters of phenol (A), glycerol (B), and 2-naphthol-6-SO_3Na (C) by green bean extract. pI is the negative $\log_{10}$ of molar inhibitor concentration.

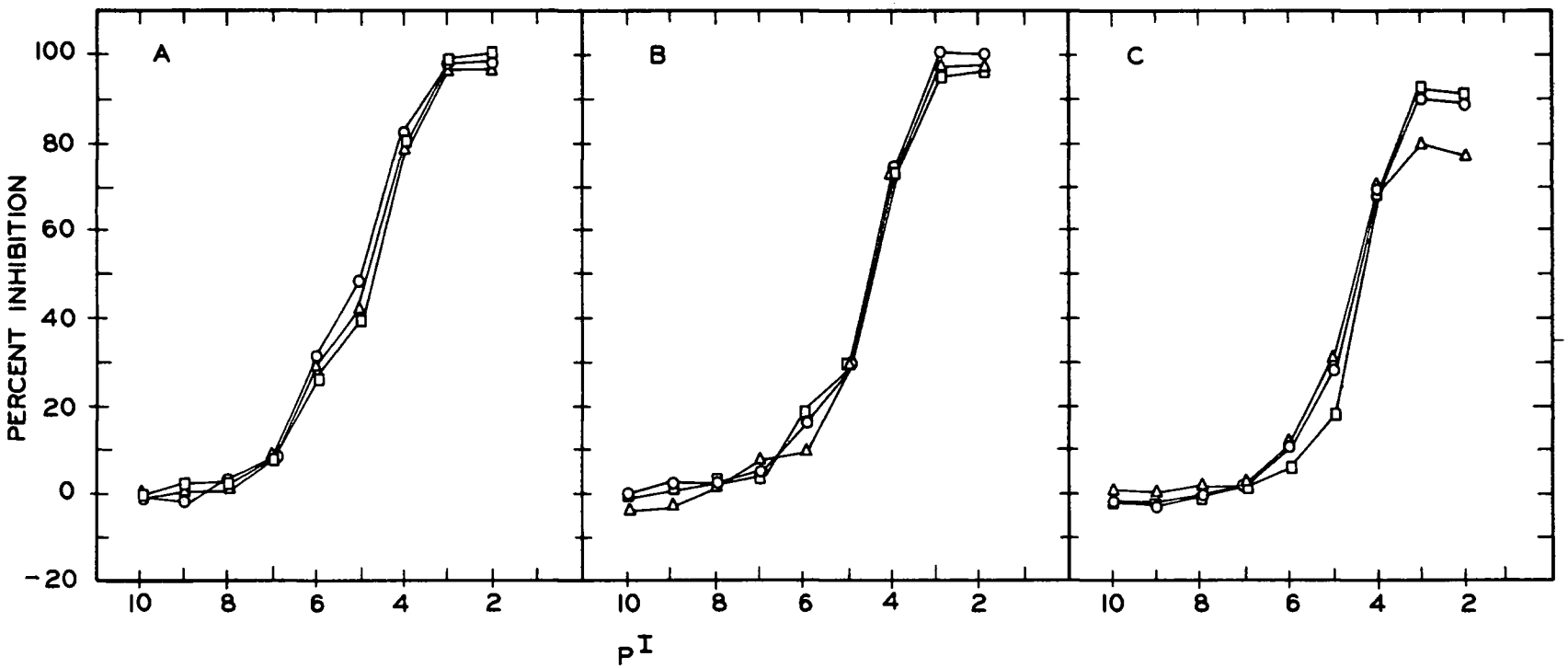


Figure 5. Inhibition by various DFP concentrations of the hydrolysis of acetyl (△——△), propionyl (○——○), and butyryl (□——□) esters of phenol (A), glycerol (B), and 2-naphthol-6-SO_3Na (C) by green bean extract. pI is the negative $\log_{10}$ of molar inhibitor concentration.

in the same ratio, as shown by the nearly parallel lines in Figure 3A, but demonstrated various acyl chain length specificity with the triglycerides and naphthyl esters. The more sensitive esterase was more specific for the propionyl esters of glyceryl and 2-naphthol-6-SO_3Na than the acetyl and butyryl esters.

The inhibition curves in Figure 3 did not reach 100 percent inhibition. In Figures 3A and 3B, the curves appear to approach 100 percent inhibition at pI 1 and might have reached complete inhibition if it had been possible to obtain higher concentrations of PARA. In Figure 3C the inhibition curves for NP and NB appear to plateau at 80 to 85 percent inhibition, while that of NA appears to level off at 70 percent inhibition. This suggests the presence of a third esterase in the green bean extract that was resistant to PARA and active on the naphthyl substrates, particularly NA. Further substantiation of the presence of an OP resistant esterase in the extract is shown in Figures 4C and 5C. Figure 4C reveals an esterase specific for NP, NB, and particularly NA, which was not inhibited by TEPP at pI 3, but was inhibited at pI 2. Figure 5C indicates that the esterase was resistant to DFP at pI 2. Twenty percent of the activity toward NA and 10 percent toward NP and NB appeared to be due to this esterase. Since 100 percent inhibition was reached in Figures 4A, 4B, 5A, and 5B, this resistant esterase did not hydrolyze the phenyl esters or the triglycerides.

Norgaard (1968) reported an arylesterase in the pea active toward NA, but not NP or NB, which was not inhibited by DFP, but was inhibited by PARA and TEPP. Two esterases in the carrot were found to be resistant to OP inhibitor (Carino, 1968). One of these esterases was resistant to PARA, but inhibited by DFP and TEPP, and hydrolyzed PA and NA. The other esterase was resistant to DFP and TEPP, but inhibited by PARA and hydrolyzed the naphthyl esters, but not the phenyl esters and triglycerides. Both of these enzymes were classified as arylesterases (Carino, 1968). The OP resistant esterase of green bean extract has substrate specificity similar to the resistant esterases of the pea and carrot in that all the OP resistant esterases appear to hydrolyze the naphthyl esters. However, inhibitor specificity of the OP resistant esterase of beans is different, since the bean esterase was resistant to both PARA and DFP, but inhibited by TEPP.

Figures 4 and 5 do not show the double sigmoid curves as found in Figure 3. This indicates that TEPP and DFP were not as selective inhibitors of the OP sensitive esterases of the bean extract as PARA. Figure 4A and B reveal that nearly 100 percent inhibition was reached with the phenyl esters and triglycerides at pI 4 of TEPP, while the same level of inhibition required a ten-fold higher concentration of DFP (Figure 5A and B). Therefore, the OP sensitive esterases of the bean extract appear to be more sensitive

to TEPP than DFP. Since the hydrolysis of NB was more resistant to lower concentration of OP inhibitors than NA and NP (Figures 4C and 5C), the more OP sensitive esterase of the bean extract does not hydrolyze this substrate as readily as it hydrolyzes the shorter acyl chained naphthyl esters.

By means of the inhibition and substrate specificity studies, it was possible to distinguish three different esterases. However, other esterases having the same substrate and inhibitor specificities could exist in the extract and would not be demonstrated by this type of data. Electrophoretic studies might reveal additional esterases.

Of the three esterases found in the water extract of green beans, two appear to be active toward the phenyl and glyceryl esters, while all three were active toward the naphthyl esters. The esterase most sensitive to the OP compounds was more active on the phenyl esters, than on the glyceryl and naphthyl esters, while the less sensitive esterase preferred the glyceryl esters. These esterases, which were inhibited by the OP compounds would be classified carboxylesterases, according to the classification for animal esterases. The esterases resistant to PARA and DFP was specific for the naphthyl esters, being more active on the acetyl ester than on the propionyl or butyryl esters. This suggests that this esterase was similar to the arylesterase of animal tissue

(Aldridge, 1953; Augustinsson 1959). Since this esterase did not hydrolyze the phenyl esters (Figures 3A, 4A, and 5A), it differs in substrate specificity from animal arylesterase as defined by Augustinsson (1958, 1961).

The use of the classification system of Augustinsson (1961) for animal esterases to classify plant esterases is conceivable. Exceptions to the definitions do occur. Norgaard (1968) found evidence for as many as six esterases in peas, five of which were carboxylesterases. The sixth was proposed as an arylesterase. Although it was not inhibited by DFP, it was inhibited by PARA and TEPP. Carino (1968) reported six esterases in carrots two of which showed resistance to some OP compounds. One, which hydrolyzed PA and the naphthyl esters, was resistant to PARA, but was inhibited by DFP and TEPP. A second esterase of the carrot was not inhibited by pI 4 to pI 2 of DFP, hydrolyzed TEPP at pI 3, but was inhibited by pI 2 TEPP. The esterase found in beans that was specific for the naphthyl esters was likewise, resistant to two OP compounds, but inhibited by a third OP inhibitor. Aldridge (1954) reported a similar situation with arylesterase from rat pancreas which was not inhibited by three OP compounds, but which was inhibited by paraoxone. This inhibition was found to be reversible.

As more esterases are purified and the physiological activities

are investigated, it may be possible to develop more appropriate systems of nomenclature and classification.

SUMMARY

An investigation to differentiate and classify the esterases of an aqueous extract of green beans was made. The esterases were found to hydrolyze the acetyl, propionyl, and butytyl esters of phenol, glycerol, and 2-naphthol-6-SO_3Na. They failed to hydrolyze triolein, and the longer chain 2-naphthol-6-SO_3Na esters, indicating that no long chain hydrolyzing esterases or lipases were present. The bean extract did, however, hydrolyze the choline esters slightly. This activity was probably due to one of the other esterases, since carboxylesterases have been reported to hydrolyze choline esters. The pH for optimum activity of the bean esterases on five substrates was 7.2.

A range of concentrations of PARA, TEPP, and DFP had various effects on the hydrolysis of the substrates by the extract. Two esterases active on all three groups of substrates were inhibited by different concentrations of the OP compounds. A third esterase, active on the 2-naphthol-6-SO_3Na esters, most specifically the acetyl ester, was resistant to PARA, DFP, and to TEPP at pI 3. It was, however, inhibited by pI 2 of TEPP.

According to the classification used for animal esterases, the two OP sensitive esterases would be designated carboxylesterases, while the resistant esterase would be grouped with the arylesterases.

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