QUANTITATIVE ISOLATION OF VICINE FROM FABABEAN PROTEIN CONCENTRATE AND THE INVOLVEMENT OF FREE RADICALS IN THE BIOPHYSIOCHEMICAL EFFECTS OF DIETARY VICINE IN THE CHICKEN

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by

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

DEDICATED TO MY GRANDMOTHER, Z. KAMPI

ABSTRACT

Experiments were conducted to (1) develop a method for isolating vicine from fababean protein concentrate (FBPC) or dehulled ground fababeans, in such quantities as to permit animal feeding trials; (2) investigate the biophysiochemical effects of dietary vicine in chicks and laying hens; and (3) establish the mode of action of vicine in eliciting its effects in animals.

Crude vicine (vicine + convicine, 5.7:1) was extracted from FBPC with either 10.5 (74:30:1) or 7.5 (25:11.5:1) volumes of acetone/water/3N NaOH mixtures. The extract was concentrated to 0.07-0.08 volumes in a steam-heated cyclone evaporator, and then stored to crystallise at $0^{o}C$. The crystallite was washed successively with water, ethanol and acetone, and then dried to constant weight at $60^{o}C$. Attempts to use dehulled ground beans instead of FBPC were unsuccessful because the sample was difficult to filter, the yield extremely low and the product was contaminated with a sticky substance. Crude vicine was separated into vicine and convicine by recrystallisation, and several properties were determined on these compounds. Crude vicine was added to a normal broiler starter diet at 0, 0.25, 0.5, 1.0 or 2.0% levels, and the diets were fed to 7-day old chicks ad libitum for 2 weeks. In addition crude vicine was added to a chicken breeder diet at 0, 0.5 or 1.0% levels in one experiment; and at 0 or 1.0% level in two other experiments. The diets were fed to laying hens ad libitum or pair-fed. Reduced divicine was prepared from vicine by acid hydrolysis and its spectral, stability and some biochemical properties were investigated. The production of free radicals during the autoxidation of reduced divicine was investigated

utilising the reduction of cytochrome (cyt C) and nitroblue tetrazolium (NBT), or using electron spin resonance spectroscopy.

The physical (e.g. crystalline, melting point), spectral and stability characteristics indicated that the compounds isolated were vicine and convicine. However, the amino acid chromatography method revealed that the "pure" vicine and convicine were 97 and 91% pure, respectively, and each was contaminated by the other. The compound obtained after acid hydrolysis of vicine was confirmed to be reduced divicine from its spectral, stability and reducing properties. Increasing levels of dietary vicine, particularly above 1%, depressed chick growth; this was not due to lowered feed intake. In the laying hen, both 0.5 and 1.0% dietary vicine led to reduced egg and yolk masses ($P<0.05$), due mainly to lower weights rather than production rate.

In the other two experiments where 0 or 1.0% dietary vicine was fed other parameters were investigated. In addition to lowered egg and yolk masses, vicine led to increased yolk membrane fragility and the incidence of yolk blood spots; and reduced fertility and hatchability of the eggs.

Vicine-fed birds had elevated levels of plasma lipids, lipid peroxides and erythrocyte hemolysis in vitro, and depressed hematocrit and the ratio of plasma vitamin E/lipid levels ($P<0.05$). Dietary vicine resulted in lower red blood cell (RBC) hemoglobin but elevated reduced glutathione (GSH) levels and superoxide dismutase (SOD) activity ($P<0.05$). It also led to higher liver weights, and lipid peroxide and GSH levels ($P<0.05$), but the effect on liver lipid levels depended on the assay method; vicine-fed birds had higher ($P<0.05$), but lower ($P>0.05$) liver lipid levels when

wet or lyophilised livers were employed in the assay, respectively. Liver protein levels were slightly depressed (P 0.05) in vicine-fed birds. Activities of liver glutathione peroxidase (GSH-px), catalase and SOD were lower (P 0.05) in the vicine-fed birds. All the above effects were not due to reduced feed consumption. Vitamin E supplementation at levels forty times higher than the NRC recommendations for laying hens, alleviated the vicine-mediated reduction in egg fertility and hatchability, it also reduced liver weight and lipid levels and restored the enzyme activities of liver GSH-px and SOD, but not catalase or the other parameters observed in the laying hen. In addition exogenous glucose in the incubation medium inhibited the spontaneous hemolysis in vitro of RBC from vicine-fed birds.

Reduced divicine slowly autoxidised in acid but rapidly in basic media equilibrated with air but not nitrogen. In solutions equilibrated with air, reduced divicine reacted with GSH to form a product that absorbed strongly at 305 nm. In the presence of GSH, a mixture of cyt C and reduced divicine showed decreased absorbance at 240 and 550 nm, but an increase at 305 nm. Methemoglobin (MetHb) was reduced to hemoglobin (Hb) by reduced divicine, and both MetHb and Hb enhanced the autoxidation of divicine. The autoxidation of divicine was initially inhibited, but finally enhanced by SOD or catalase, while H_2O_2 inhibited the latter and not the initial part of the autoxidation. The effect of H_2O_2 was alleviated by catalase. Reduced divicine reduced both cyt C and NBT. However, unlike NBT, a minor part of the cyt C reduction was susceptible to SOD or catalase inhibition. In addition other proteins like bovine serum albumin (BSA), Hb, MetHb, alcohol

dehydrogenase and ovalbumin at low concentrations inhibited the oxygen-dependent reduction of NBT by divicine.

Considering this data and that in published literature it may be concluded that vicine is hydrolysed to its aglycone, divicine at a site not specifically known (digestive tract, plasma or liver). The increased activity of SOD in the RBC would indicate a high concentration of O_2^- in plasma, implying that most of the autoxidation of divicine occurs in plasma and therefore vicine is either hydrolysed there or before it reaches the circulation. The divicine produced can then condense directly with some biological compounds like GSH, or it autoxidises in the alkaline plasma to produce O_2^-. The O_2^- can them dismutate to H_2O_2 and finally produce $^{\bullet}OH$, which is the most potent oxidant of the known free radicals. The cells would not be able to protect themselves due to the lowered activity and/or levels of the liver enzymes, GSH-px, SOD and catalase. The increased levels of liver and RBC GSH would indicate an inhibition of its utilisation, with little effect and/or increase in its production. Both effects of divicine lead to oxidation of the biological molecules, causing lipid peroxidation and RBC hemolysis. This may cause destruction of the lipid transport proteins or alter the lipids such that they are not transportable to the ovum, but accumulate in plasma. Finally, vitamin E can protect the chicken against some, but not all vicine-mediated effects. Therefore other nutrients may be involved in the action of vicine.

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Sources of the chemicals utilised in the studies

Chemical Name	Chemical Company
Triton X-100 scintillation grade	Amersham Corp.
H_2O_2	Baker Chemical Company
NaCl	" " "
Sodium citrate	" " "
TCA (Trichloroacetic acid)	" " "
$NaH_2PO_4.H_2O$	" " "
Tris buffer	" " "
KH_2PO_4	" " "
$FeCl_3.6H_2O$	" " "
Metaphosphoric acid (glacial)	" " "
$CuSO_4.5H_2O$	BDH Chemical Company
EDTA disodium salt	Fisher Scientific Company
n-propanol	" " "
$FeSO_4.7H_2O$	" " "
Na_2HPO_4	" " "
K_2HPO_4	" " "
Sodium cyanide	" " "
Na_2CO_3	" " "
NaOH	" " "
Glucose (dextrose)	" " "
H_2SO_4	" " "
$KMnO_4$	" " "
Antifoam B	" " "
HCl	" " "

Continued .....

Sources of the chemicals utilised in these studies (Continued)

Chemical Name	Chemical Company
Folin - Ciocalteu (phenol) reagent	Harleco Company
TBA (2-thiobarbituric acid)	ICN Pharmaceuticals Inc.
Sodium azide	Matheson, Coleman & Bell
Methionine	Nutritional Biochem. Corp.
Riboflavin	" " "
GSH (reduced)	Sigma Chemical Company
GSSG (ox) (No. G4501) (grade III)	" " "
5,5^{1} dithiobis-(2-nitrobenzoic acid)	" " "
α, α^{1}-dipyridyl (∂, ∂^{1}-bipyridine)	" " "
d$\ell\alpha$-tocopherol	" " "
BHT (Butylated Hydroxytoluene)	" " "
Nitro Blue Tetrazolium	" " "
NADPH	" " "
Hemoglobin Standard Kit:	" " "
- Hemoglobin Standard	" " "
- Drabkin's Reagent	" " "
- Brij 35 soln.	" " "
Penicillin-G	" " "
NaK tartrate	" " "
Catalase from bovine liver Stock #C-10	" " "
Cytochrome-C, Type VI	" " "

Continued .....

Sources of the chemicals utilised in these studies (Continued)

Chemical Name	Chemical Company
Superoxide Dismutase, Type I from bovine blood	Sigma Chemical Company
Hemoglobin No. H-2500 (from beef blood)	" " "
Methemoglobin No. m-9250 (from beef blood)	" " "
Bovine albumin, fraction V	" " "
Alcohol dehydrogenase (from yeast)	" " "
Ovo albumin, Grade V	" " "
Glutathione, reduced form	" " "
Acetone	Standard Chemical Company
Ethanol	" " "

List of Abbreviations

AA - Ascorbic acid

α - Specific rotation

APH - Acetylphenylhydrazine

DA - Dialuric acid

DETAPAC - Diethylenetriamine pentaacetic acid

DIL (5x) - SAT diluted five times etc.

DMPO - 5,5-dimethyl-1-pyrroline-N-oxide

EDTA - Ethylenediamine tetraacetic acid

e^- - Electron

ε - Molar absorption coefficient

esr - Electron spin resonance

FBPC - Fababean protein concentrate

4-POBN - α-4-pyridy-1-oxide N-tert-butyl nitrone

G-LC - Gas-liquid chromatography

G-6-P - Glucose-6-phosphate

G-6-PD - Glucose-6-phosphate dehydrogenase

GSH - Reduced glutathione

GSSG - Oxidized glutathione

GS* - Free radical of oxidized glutathione

GSSG-red - GSSG-reductase

Hb - Hemoglobin

HCl - Hydrochloric acid

HMDA - Hexamethyldisilazane

λ - Wavelength

Continued .....

List of Abbreviations (Continued)

λ max - Wavelength at which maximum absorption occurs

λ min - Wavelength at which minimum absorption occurs

L-Dopa - L-3,4-dihydroxyphenylalanine

$L^{\cdot}$ - Alkyl radical

$LO^{\cdot}$ - Alkoxy radical

$LOO^{\cdot}$ - Hydroperoxy radical

ME - Metabolisable energy

MetHb - Methemoglobin

NADH, NAD^{+} - Reduced and oxidized Nicotinamide Adenine (diphosphopyridine nucleotide)

NADPH, $NADP^{+}$ - Reduced and oxidized Nicolinamide Adenine Dinucleotide 2^{1}-phosphate

nmr - nuclear magnetic resonance

NSHA - Non spherocytic hemolytic anemia

$^{1}O_{2}$ - Singlet oxygen

O_{2}^{-} - Superoxide anion or radical

O_{3} - Ozone

OD - Absorbance

ΔOD - Change in OD

$^{\cdot}OH$ - Hydroxyl radical

OxyHb - Oxyhemoglobin

PCMB - p-chloromercuric benzoic acid

PUFA - Polyunsaturated fatty acids

PVP - Polyvinylpyrrolidone

Continued .....

List of Abbreviations (Continued)

RBC - Red Blood Cells

RH_2 - Any reduced compound with two hydrogens capable of being oxidized

$RO_2^{\bullet}$ - Peroxy radical

SAT - Saturated solution of divicine or vicine

SH - thiol

6-ADA - 6-aminodopamine

6-OHDA - 6-hydroxydopamine

6-PGD - 6-phosphogluconate dehydrogenase

SOD - Superoxide dismutase

TCA - Trichloroacetic acid

TLC - Thin layer chromatography

TMCS - Trimethylchlorosilane

TMPO - 2,5,5-trimethyl-1-pyrroline-N-oxide

TMS - Trimethylsilyl

TPN - Triphosphopyridine nucleotide

UV - Ultraviolet

INTRODUCTION

Fababeans have been cultivated for more than 3,000 years and have been grown and utilised as a food in many countries, particularly the Near East, Mediterranean and North African states. However, fababeans grown in Europe are used mainly as a livestock feed. In North America, particularly Canada, fababeans are a promising crop both agronomically and economically to alleviate the heavy dependence on imported soybeans as a protein supplement in livestock feeds. Fababeans are also a potential source of protein for man, particularly in developing countries where livestock products are limited.

In spite of their high nutritional qualities (e.g. high protein content (25-35%) with a good balance of amino acids, although deficient in S-amino acids particularly methionine), fababeans contain several antinutritional factors. These antinutritional factors consist of thermolabile and thermostable compounds. The thermolabile factors consist of several polyphenolic compounds, lectins and trypsin and/or general and amylase inhibitors, and are equally distributed between the hull and cotyledon except the lectins which do not occur in the hull. The thermolabile antinutritional factors found in the cotyledon are more specifically associated with the protein fraction. The major thermolabile factors in the hull are the polyphenolic compounds including condensed tannins, which complex with a lot of dietary nutrients, particularly proteins and amino acids rendering them unavailable to the animal. The effects of the heat-labile factors are easily alleviated by heat treatment, in addition dehulling can remove those factors restricted to the hull, while tannins can be selectively bound by PVP or avoided