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DETERMINATION OF FOOD PROTEIN QUALITY BY A NEW BIOLOGICAL APPROACH. I. BIOLOGICAL VALUE (BV) OF PEA PROTEIN AND EFFECT OF SYNTHETIC AMINO ACID SUPPLEMENTS DESIGNED TO ACHIEVE ITS MAXIMUM

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Key words: essential amino acids (EAA), non-essential amino acids (NEAA), N-retention, biological value (BV)

The objective of this part of the study was to determine biological value (BV) of pea protein for growing rats. Also, to compare the N-retention responses of the rats to supplementation of the pea protein with: (1) the same protein, (2) the mixture of essential and non-essential amino acids formulated to reproduce the pea protein, and (3) the mixture of essential amino acids only, formulated as above. The supplements were fed in the quantities defined by BV and equal to: (1-BV)/BV.

A Polish cultivar of peas (*Cyrkon*), was a source of protein. Nutritional experiments were conducted using albino rats fed semi-purified diets and the N-balance method of Thomas-Mitchell, as described by Eggum [1973].

The relationship between increasing intake of pea protein and 24-h nitrogen retention in growing rats was linear only at the protein intake ranging from 0.50 g to 0.84 g/24 h. Pea protein supplementation with the same protein significantly increased ($p<0.05$) N-retention by 50.3 mg/kg $W^{0.75}$, thus giving the expected rise up to the maximum value of N-utilization (BV=1). It was also evidenced that the pea protein supplement can be replaced by the mixture of essential and non-essential amino acids formulated to reproduce the pea protein, and the mixture of essential amino acids only, formulated as above, without affecting N-balance.

INTRODUCTION

Identification of essential amino acids (EAA), limiting nutritional value of a protein in human nutrition still remains a matter of debate [FAO/WHO, 1991]. Currently used indices of protein nutritional value, despite their indisputable practicality, are invariably the values which are a function of the first limiting amino acid, and do not account for nutritional value of remaining amino acids. Recently, the chemical score (CS) method as the basic tool for evaluation of protein quality, was modified by FAO/WHO experts [1991] by introducing a correction for true digestibility (PDCAAS = CS x TD) of a protein under examination (PDCAAS – Protein Digestibility-Corrected Amino Acid Score). However, such manner of evaluation of protein nutritional value raises severe doubts. The main concern is reliability of the recommended amino acid pattern which was advanced in the eighties on the basis of trials on a limited number of 2-year-old children. Also, true protein digestibility, determined along the whole length of the alimentary tract, bears an error resulting from endogenous losses of amino acids in the large intestine. Moreover, the validity of truncation the PDCAAS values to 100% is being questioned [Schaafsma, 2000; Tome & Bos, 2000; Young & Borgonha, 2000]. Given the above-mentioned shortcomings of the currently recommended [FAO/WHO, 1991] method of food protein evaluation (*i.e.* PDCAAS), the objective of our study was to develop an alternative biological method avoiding these shortcomings and allowing simultaneous determination of the extent and order of EAA limitation in food proteins. The new method is based on a mathematical model of nitrogen metabolism, which would fully account for nutritional role of *all essential amino acids*, as factors determining potential nutritional value of food proteins. This approach was first applied to study the limiting amino acids of rumen microbial protein by Storm and Orskov [1984].

MATERIALS AND METHODS

Theoretical considerations. The following three assumptions, resulting from curvilinear relationship between protein intake and its utilization (BV) by growing rats, were adopted. First, the biological value (BV) of a given protein is reliable only when it has been determined in the linear range of the curve describing relationship between protein intake and nitrogen retention. Second, the difference between biological value of the given protein under examination (BV<1) and maximum value of this index (BV=1) is a function of deficit of the first limiting amino acid in this protein. For a protein with a given biological value (BV<1), the amount of the same protein supplement (PS, %), which would be theoretically required to cover deficit of the first limiting amino acid and then attain maximum N-utiliza-

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tion (BV=1) is defined by the equation: PS = (1-BV) / BV. Third, the protein supplement (PS, %), calculated as above, may be replaced by synthetic amino acid supplements of either NEAA+EAA or EAA only, designed to reproduce the given protein amino acid composition; additionally assuming that absorption of synthetic amino acids in the rat small intestine equals 1. Consequently, according to the first and second assumption, replacement of PS with the above amino acid mixtures (reproducing its composition), should allow, theoretically, for an increase in N-utilization to its maximum value (BV=1).

Experimental animals and composition of semi-purified diets. Experiments were conducted on male albino rats obtained from the Department of Animal Nutrition of the Institute of Animal Production in Kraków. The animals were 5-week-old and weighed 90–110 g. The study was carried out in compliance with ethical requirements [Close *et al.*, 1996, 1997]. Animals were kept individually in digestibility cages in a room maintained at constant temperature and humidity, under 12 h/12 h light/dark cycle. During adaptation period (7 days), the animals had constant access to a standard rodent commercial feed (GLM-1) and drinking water.

During experiments, the animals were fed the semi-purified diet, formulated according to Eggum [1973]. Seeds of pea (cv. *Cyrkon*), derived from the Institute of Plant Breeding and Acclimatization (Oleśnica Mała, Poland), were ground using a laboratory grinder Kniftec 1095 (Foss--Tecator) and autoclaved (121°C, 15', 1 atm.). The seeds were the sole source of dietary protein. The diet contained also (%): sucrose (20), soybean oil (5), cellulose (Whatman CF11; Sigma Aldrich, Poznań, Poland,) (5), mineral mixture (4) and vitamin mixture (1). Composition of mineral and vitamin mixtures was also adopted from Eggum [1973]. The diets were made up to 100% with wheat starch (DIA-CEL Łódź). The gross chemical composition of pea seeds and amino acid composition of pea protein are given in Table 1 and 2.

Experimental design and feeding of animals. Three experiments (I, II and III) were carried out to verify the theoretical assumptions of the present approach.

TABLE 1. Gross chemical composition of pea seeds cv. *Cyrkon* (in % d.m.).

Protein	Ether extract	Carbohydrates	Crude fiber	Ash
22.8	1.23	55.29	5.15	3.53

Experiment I was designed to determine the efficiency of utilization (BV) of pea protein, by growing rats, over a wide range of the protein intakes, in order to verify the first assumption. Fifty growing rats were randomly assigned to 5 experimental groups (10 rats each) and fed five semi-purified diets providing graded levels of pea protein (%): G_1 – 5.0, G_2 – 7.5, G_3 – 10.0, G_4 – 12.5 and G_5 – 15.0, as the only source of nitrogen.

Experiment II was designed to determine the effect of supplementing the basal diet with pea protein (PS, %) on nitrogen retention in growing rats, in order to verify the second assumption. Twenty growing rats were randomly assigned to 2 experimental groups (10 animals each) and fed two semi-purified diets: basal diet (G_1 – 5.0% of protein), and basal diet with pea protein supplement (G_1+PS; giving 8.3% of protein), the latter calculated according to the second assumption.

Experiment III was designed to determine the effect of the NEAA+EAA and the EAA supplements (reproducing the composition of pea protein) on nitrogen retention in growing rats, in order to verify the third assumption. Forty growing rats were randomly assigned to 4 experimental groups (10 animals each) and fed four semi-purified diets: basal diet (G_1), basal diet with pea protein supplement (G1+PS) and basal diets supplemented with amino acid mixtures (G_1+NEAA+EAA) or (G_1+EAA). Amino acid supplements were prepared from synthetic amino acids (Sigma-Aldrich, Poland) supplied as L forms. The composition of NEAA+EAA and EAA only supplements is given in Table 3.

Nitrogen balance and preparation of biological material for analyses. Nitrogen balance (nitrogen retention) was measured in growing rats using Thomas-Mitchell method [Eggum, 1973]. N-balance experiments, conducted after adaptation period, consisted of 4 days of the initial period and 5 days of the experimental period. The rats were weighed three times: at the beginning of initial period (day 1), and at the beginning and end of experimental period (day 5 and 9). During experiments, level of feeding was limited to 10 g of semi-purified diet per rat per 24 h. Excreta (feces and urine) were collected quantitatively and pooled for each individual. Fecal samples were frozen and freeze-dried using an Alpha 1–4 (Christ) freeze-drier. Urine samples were adjusted to constant volume (200 mL) with 5% sulfuric acid. Refused diets were freeze-dried (as described above) and weighed.

TABLE 2. Amino acid composition of pea seeds cv. *Cyrkon* in comparison with FAO/WHO* standard.

Amino acids	Ala	Arg	Asp	Glu	Gly	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Pro	Thr	Ser	Val
Content in g/100 g of product	0.095	0.163	0.271	0.465	0.095	0.047	0.097	0.146	0.127	0.022+0.034	0.096+0.073	0.138	0.102	0.104	0.105
Content in g/ 100 g of protein	4.16	7.16	11.87	20.40	4.15	2.06	4.23	6.39	5.56	0.97+1.50	4.21+3.21	6.07	4.48	4.56	4.61
FAO/WHO* standard in g/100g of protein	----	----	----	----	----	1.9	2.8	6.6	5.8	2.5	6.3	----	3.4	----	3.5

* Standard of amino acid requirement in preschool children (2–5 years old) according to FAO/WHO [1991].

TABLE 3. Composition of amino acids of experimental diets.

Amino acids NEAA+EAA	NEAA	EAA	g/ kg diet
Asp	Asp	---	3.72
Thr	---	Thr	1.41
Ser	Ser	---	1.43
Glu	Glu	---	6.39
Pro	Pro	---	1.91
Gly	Gly	---	1.30
Ala	Ala	---	1.30
Val	---	Val	1.45
Ile	---	Ile	1.33
Leu	---	Leu	2.01
Tyr	---	Tyr	1.01
Phe	---	Phe	1.32
His	---	His	0.65
Lys	---	Lys	1.75
Arg	Arg	---	2.25
Cys	---	Cys	0.47
Met	---	Met	0.31

Chemical analyses. Gross composition of Cyrkon seeds was determined using standard methods recommended by the Association of Official Analytical Chemists [AOAC, 1995]. Amino acid composition of pea protein was determined in seed samples hydrolyzed in 6N HCl (24 h, 110°C). In addition, separate samples were oxidized [Moore, 1963] to convert methionine and cysteine into methionine sulphone and cysteic acid, respectively. Amino acid composition of the hydrolyzates was determined in the Institute of Animal Production (Kraków, Poland) on a Carlo Erba 3A 29 automatic amino acid analyzer according to the classical ion-exchange procedure of Spackman *et al.* [1958]. The material obtained during N-balance experiments, *i.e.* diet refusals, freeze-dried feces and urine samples were analyzed for total-N using the Kjeldahl method [AOAC, 1995].

Statistical analysis. The relationship between increasing intake of protein N of peas and its retention in rats was analyzed using a curvilinear regression model with an inflection point [Mercer, 1982; Remmenga *et al.*, 1997], in order to determine the linear range of the regression curve; the Statistica 5.0 programme was used. The data of N-balance experiments were subjected to one-way ANOVA. Significance of differences treatment between means was tested using Tukey's test at the $p<0.05$ level of probability.

RESULTS

Experiment I

The efficiency of protein utilization (BV) decreased with increasing intake (0.50, 0.75, 1.00, 1.25 and 1.50 g per 24 h) of pea protein in semi-purified diets (Table 4). For three lower intake levels, BV was similar, ranging from 0.598 to 0.606, and the values did not differ significantly ($p>0.05$). On the other hand, at two highest intake levels, BV was significantly lower ($p<0.05$) ranging from 0.502 to 0.518. True digestibility (TD) of pea protein was independent of its intake level ($p>0.05$), averaging 0.945. The pattern of net protein utilization (NPU) changes corresponded with that described for BV.

It was also shown that, according to the low of diminishing returns, nitrogen retention significantly rose ($p<0.05$) with increasing dietary protein intake from 84.6 mg/kg $W^{0.75}$ for the lowest intake level (0.50g of protein/ 24 h) to 214.4 mg/kg $W^{0.75}$ for the highest protein intake (1.50 g/24 h). The above relationship between the model protein intake in semi-purified diets (x: g/24 h) and nitrogen retention in growing rats (y: mg/kg $W^{0.75}$) is described by the curvilinear regression equation (Figure 1):

$$y = -4.72 + 178.69x - 78.08 (x-0.84) (x>0.84)$$

where: $x > 0.84$ – logical condition, equal to 0 or 1; $R^2 - 0.905$; $n = 36$.

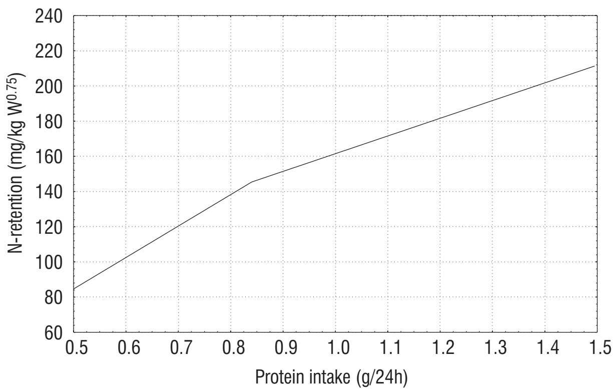

FIGURE 1. The relationship between dietary protein intake and N-retention in growing rats.

Experiment II

The amount of pea protein supplement (PS, %), required to attain an increase in N utilization up to maximum value (BV=1) was calculated from the formula $PS=G_1 \times (1-BV)/BV$; adopting the pea protein BV= 0.600 (Table 4). Consequently, the G_1 diet, containing 5% of

TABLE 4. Biological value (BV), true digestibility (TD) and net protein utilization (NPU), and N-retention for diets with increasing contents of *Cyrkon* pea protein.

Diet	BV	TD	NPU	N retention (mg/ kg $W^{0.75}$)
5% of protein (0.5 g of protein/ 24 h) G_1	0.600±0.070 [a]	0.954±0.042 [a]	0.607±0.083 [a]	84.6±11.3 [a]
7.5% of protein (0.75 g of protein/ 24 h) G_2	0.598±0.096 [a]	0.964±0.021 [a]	0.575±0.083 [a]	129.3±14.0 [b]
10% of protein (1 g of protein/ 24 h) G_3	0.606±0.049 [a]	0.953±0.023 [a]	0.573±0.038 [a]	164.9±8.9 [c]
12.5% of protein (1.25 g of protein/ 24 h) G_4	0.502±0.048 [b]	0.927±0.008 [a]	0.465±0.044 [b]	178.0±19.8 [d]
15% of protein (1.5 g of protein/ 24 h) G_5	0.518±0.032 [b]	0.929±0.010 [a]	0.480±0.018 [b]	214.4±9.8 [e]

The values in columns marked with different letters differ significantly ($p<0.05$).

pea protein and providing 0.50 g protein per 24 h, was supplemented with 0.33 g of the same protein (PS=0.50 x (1-0.6)/0.6), increaing the total protein intake to 0.83 g/24h. The N-retention in rats (Table 5), fed G_1 diet (0.5 g of protein/24 h) was 84.6±11.3 mg N/kg $W^{0.75}$, while G_1 diet supplemented with pea protein (G_1+PS) significantly ($p<0.05$) increased N retention to an average of 134.9±9.4 mg N/kg $W^{0.75}$. The difference between these values, equal to 50.3 mg N/kg $W^{0.75}$, was the expected increase in N retention, giving the maximum value of protein utilization (BV=1), thus confirming the second assumptions of this study.

Experiment III

The substitution of pea protein (PS: 0.33 g per 24 h) with either the NEAA+ EAA or EAA only supplements (reproducing the amino acid composition of PS), gave N-retention reaching 139.8±17.3 mg/kg $W^{0.75}$ and 134.2±23.8 mg/kg $W^{0.75}$, respectively (Table 5). Moreover, no significant differences in N retention ($p>0.05$) between rats fed G_1+ PS, G_1+(NEAA+EAA) and G_1+(EAA) diets were found. Thus it was possible to substitute synthetic amino acids (either NEAA+EAA or EAA) for pea protein, without affecting N-retention and attaining maximum value of protein utilization (BV=1). These results confirm the third assumptions of this study.

TABLE 5. N-retention level for basal diet (G_1) and diets with different supplements: the protein (G_1+PS), sum of synthetic amino acids (G_1+NEAA+EAA), essential amino acids (G_1+EAA).

Diet	N retention (mg/ kg $W^{0.75}$)
G_1	84.6±11.3^a
G_1 + PS	134.9±9.4^b
G_1 + NEAA + EAA	139.8±17.3^b
G_1 + EAA	134.2±23.8^b

The values in columns marked with different letters differ significantly ($p<0.05$).

DISCUSSION

Pea as a source of protein

Chemical composition of seeds of edible pea cv. *Cyrkon* (Table 1), did not differ from the values reported by other authors [Waszkiewicz-Robak, 1993; Kozłowska & Troszyńska, 1995; Lampart-Szczapa, 1997]. Also, amino acid composition of this protein (Table 2) was typical [Eggum *et al.*, 1989; Jasińska & Kotecki, 1993; Kunachowicz *et al.*, 1998], with characteristic low content of sulfur amino acids (Met+Cys: 2.47 g/100 g of protein). Thus, it is not surprising that its biological value (BV) determined in the present study was relatively low (BV=0.600±0.070) and comparable with the value observed earlier [Eggum *et al.*, 1989]. True digestibility of pea protein (TD=0.954±0.042) and net protein utilization (NPU=0.607±0.083) were also in agreement with earlier observations [Eggum *et al.*, 1989].

The choice of pea seeds, with the relatively low BV value, as a source of protein, resulted from methodological reasons. Namely, pea protein supplement amounted to 66% of the same protein content in G_1 diet (PS = (1-BV)/BV, *i.e.* (1-0.6)/0.6 = 0.66). It enabled us to add a relatively large amount of pea protein, and to reproduce its composition with considerable amounts of synthetic amino acids.

Verification of theoretical assumptions

The relationship between the level of protein intake by growing rats and N-retention (Table 4; Figure 1), illustrates the law of diminishing returns [Marcer, 1982]. It this study it is characterized by a linear increase up to certain protein intake level (*i.e.* requirement), and subsequent curvilinear bending, resulting from gradual drop in the efficiency of utilization of a protein intake unit. Similar relationships were observed as typical, in growing rats, in a number of comparable trials [Heger & Frydrych, 1985; Gahl *et al.*, 1991]. However, it is worth emphasizing that there are at least several different mathematical models used to describe the above relationship and allowing determination of protein requirement. Frequently, they include a linear-linear model with an inflection point, in which relationship between protein intake and N retention is linear below the requirement, and when it is exceeded, further increase in protein intake does not influence retention [Lewis, 1992]. Functions more closely reflecting the actual shape of the curve of diminishing returns have also been proposed, such as an exponential model and a saturation kinetics model [Fuller & Garthwaite, 1993; Remmenga *et al.*, 1997]. Since the aim of this experiment was not to determine the protein requirement of rats but only to define linear range of the curve describing the relationship between the model protein intake and N-retention, the simplest linear model with inflection point was chosen for statistical analysis. The inflection point calculated using this model, *i.e.* 0.84 g/24 h, was, by definition, the maximum value of linear range of the curve. It implies that an increase in N-retention in growing rats responds linearly to pea protein intake only in the range from 0.50 to 0.84 g per 24 h. It is also the optimum range of pea protein intake, that satisfies the first assumptions of these studies. Consequently, the protein content in semi-purified diets used in the present study was designed not to exceed the level of 0.84 g/24 h.

Significant differences ($p<0.05$) between N retention in rats fed G_1 diet (84.6 mg/kg $W^{0.75}$) and those receiving the same diet with pea protein supplement (G_1+PS; 134.9 mg/kg $W^{0.75}$) confirmed the validity of the second assumption of these studies (Table 5). The obtained difference, *i.e.* 50.3 mg/kg $W^{0.75}$, was the expected increase in N retention up to the maximum value of pea protein utilization (BV=1).

The evident lack of significant differences ($p>0.05$) between N retention in rats fed G_1+PS, G_1+ (NEAA+EAA) and G_1+(EAA) diets (134.9, 139.8, and 134.2 mg/kg $W^{0.75}$, respectively), confirmed the validity of the third assumption. Namely, pea protein could be substituted with the amino acid mixtures, both NEAA+EAA or only EAA, without the loss of the expected increase in N-retention. Thus, the maximum value of protein utilization (BV=1) could be achieved.

The use of amino acid mixtures reproducing composition of proteins is not a new technique in nutritional studies. This technique has been widely applied for examination of amino acid requirement in higher animals, *e.g.* rats [Heger & Frydrych, 1985; Gahl *et al.*, 1991; Benevenga *et al.*, 1994] and pigs [Fuller *et al.*, 1989; Chung & Baker, 1992; Heger *et al.*, 1998; Lenis *et al.*, 1999]. It has also been commonly used to evaluate amino acid requirements in humans [Zello *et al.*, 1995; Basile-Filho *et al.*, 1998; Fukagawa *et al.*, 1998; Kurpad *et al.*, 1998; Millward *et al.*, 2000]. However, the full equivalence of supplementation with either NEAA or EAA of rat diets (Table 5) is an interesting feature of our studies. Hence, it can be presumed that under the experimental conditions applied, only EAA could be limiting. NEAA, reported as limiting in specific situations [Reeds, 2000] could not play this role. Moreover, we confirmed the opinion of Fuller *et al.* [1989] assuming 100% absorbability of synthetic amino acids, which is an important observation, since utilization of amino acids as components of different proteins and their synthetic forms can vary [Collin-Vidal *et al.*, 1994]. In contrast to our findings, Edmonds *et al.* [1987] and Edmonds & Baker [1987] observed in piglets that excessive supply of EAA led to lower feed intake and growth inhibition in these animals. More recent investigations conducted in growing rats, allowed free access to semi-purified diets, with variable amino acid compositions, point to similar effects of imbalance, and also reveal the ability of these animals to selectively choose diets with optimum AA composition [Fromentin & Nicolaidis, 1996; Hrupka *et al.*, 1997].

CONCLUSIONS

1. The relationship between the intake of pea protein and N-retention in growing rats illustrates the law of diminishing returns, and is described by the curve with an inflection point at 0.84 g/24 h, implying that N-retention in growing rats responds linearly to pea protein intake only in the range from 0.50 to 0.84 g per 24 h.

2. The amount of pea protein supplement, calculated according to the formula PS=(1-BV)/BV, covers the deficit of the first limiting amino acid in pea protein and increases the protein utilization to the maximum value (BV=1).

3. In the linear range of the relationship between the intake of pea protein and N-retention in growing rats, pea protein supplement (PS) may be substituted with either NEAA+EAA or EAA supplements (reproducing the amino acid composition of pea protein), without the loss of the expected increase in N-retention, and the maximum value of protein utilization (BV=1) can be achieved.

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NOWA BIOLOGICZNA METODA OCENY JAKOŚCI BIAŁKA ŻYWNOŚCI CZ. I. WARTOŚĆ BIOLOGICZNA (BV) BIAŁKA GROCHU ORAZ MOŻLIWOŚĆ UZYSKANIA JEJ MAKSYMALNEJ WARTOŚCI NA DRODZE PODAWANIA DODATKÓW AMINOKWASÓW SYNTETYCZNYCH

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Celem pracy było określenie efektywności wykorzystania białka przez rosnące szczury laboratoryjne oraz zbadanie wpływu wprowadzenia uzupełniającego dodatku ocenianego białka do diety podstawowej a następnie odtworzenie jego składu aminokwasami syntetycznymi i oszacowanie zmian retencji azotu.

Źródło białka stanowiła nowa polska odmiana grochu Cyrkon. Doświadczenia żywieniowe przeprowadzono metodą bilansową Thomasa-Mitchella w modyfikacji Egguma [1973] na albinotycznych szczurach laboratoryjnych szczepu Wistar. Przeprowadzono szereg doświadczeń, w których zwierzęta były karmione odpowiednimi dietami półsyntetycznymi.

W przypadku krzywej regresji, opisującej zależność pomiędzy rosnącą podażą białka w diecie i dobową retencją azotu u rosnących szczurów stwierdzono, że przebiega ona prostoliniowo tylko w zakresie podaży białka grochu w diecie od 0,50 g do 0,84 g/dobę. Wprowadzenie do diety podstawowej uzupełniającego dodatku białka (UDB), zwiększało istotnie ($p<0,05$) wielkość retencji N o 50,3 mg/ kg $W^{0,75}$, co było zakładanym wzrostem do wartości maksymalnej (BV=1), (tab. 5). Wykazano, że w prostolinowym zakresie powyższej krzywej istnieje możliwość zastąpienia UDB grochu uzupełniającym dodatkiem aminokwasów egzogennych i endogennych lub tylko egzogennych odtwarzających skład aminokwasowy tego dodatku.