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DETERMINATION OF FOOD PROTEIN QUALITY BY A NEW BIOLOGICAL APPROACH. II. IDENTIFICATION OF THE ORDER OF LIMITING AMINO ACIDS AND EXTENT OF THEIR DEFICIT IN PEA PROTEIN BY AN AMINO ACID REMOVAL TECHNIQUE

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Key words: protein quality, essential amino acids (EAA), non-essential amino acids (NEAA), amino acid removal technique, N-retention

In Part I, N-retention responses of growing rats to supplementation of pea protein, with either the same protein at a level equivalent to 0.66 of dietary protein, derived as (1-BV)/BV, or the mixture of essential amino acids, formulated to reproduce the pea protein, were equal. Having proved this, the aim of this study (Part II), was to determine the order and extent of amino acid limitation in pea protein by an amino acid removal technique. The synthetic EAA mixtures, with the same composition as that of pea protein, from which amino acids were selectively removed, were fed to rats, at the level indicated above, and the respective N-retention responses was determined. A Polish cultivar of peas (*Cyrkon*), was a source of protein. Experiments were conducted on albino rats fed semi-purified diets, using the N-balance method of Thomas-Mitchell, as described by Eggum [1973]. Removal of Met from the EAA mixture supplementing basal diet (G_1) led to significant ($p<0.05$) decrease in N retention. The same was true for Lys and or Thr. After removal of His or Phe+Tyr, N retention decreased non-significantly ($p>0.05$). On the other hand, removal of branched-chain amino acids (Leu, Ile, Val) did not affect N-retention. The obtained results allowed the identification of the order and extent of amino acid limitation (excluding tryptophane). Met, Lys and Thr were identified as the first, second and third limiting amino acid. The extent of their limitation was Met 15.4, Lys 32.2 and Thr 17.5 mg/g, in rats fed pea as the only protein source.

INTRODUCTION

We have already indicated (Part I), that currently recommended indices of protein nutritional value (*i.e.* CS and PDCAAS) [FAO, 1991], 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. However, it should be remembered that food protein is a sum of nutrients, *i.e.* individual non-essential (NEAA) and essential (EAA) amino acids; the latter determining protein quality, depending on their composition. Given the above-mentioned shortcomings of the current methods of food protein evaluation, we attempted to develop an alternative biological method avoiding these shortcomings and allowing for simultaneous determination of the order and extent 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. In Part I, N-retention responses of growing rats to supplementation of pea protein, with either the same protein, at a level equivalent to 0.66 of dietary protein, derived as (1-BV)/BV, or the mixture of essential amino acids, formulated to reproduce pea protein, were *equal*. Having proved that this was the case, the aim of this study (Part II), was to determine the order and extent of amino acid limitation in pea protein by an amino acid removal technique. The synthetic EAA mixtures, with the same composition as that of pea protein, from which amino acids were removed in turn, were fed to rats, at the level indicated above, and the respective N-retention responses were determined. It was assumed that omitting the first limiting amino acid from the EAA mixture should decrease N-retention to the level observed in animals fed the basal diet (G1), *i.e.* without the EAA supplement. Second, removal of next limiting amino acids should limit N-retention proportionally to the extent of their deficit in pea protein. Third, if N-retention is not altered by removal of a single amino acid or a group of amino acids, this should indicate that the amino acids in question were not limiting. Therefore, the proposed method should serve as a means of simultaneous identification of the order and extent of amino acid limitation in pea protein [Storm & Orskov, 1984].

MATERIAL AND METHODS

Experimental animals and composition of semi-purified diets. Experimental procedures involving laboratory rats, their housing, diet composition and feeding were the same as described in Part I of this paper [Pysz & Pisulewski, 2004].

Experimental design and feeding of animals. The experiments I and II were designed to determine the effect of selective removal of individual EAA from the EAA supplement (with the same composition as that of pea protein; derived as (1-BV)/BV), on nitrogen retention in growing rats.

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In Experiment I, thirty-six growing rats were randomly assigned to 6 experimental groups (6 rats each) and fed six semi-purified diets: G_1 – 5% of protein, G_1 + the EAA supplement (G_1+EAA) and the variants of the G_1 + the EAA supplement, from which individual EAA were removed in turn: $G_1+EAA_{(-Met)}$, $G_1+EAA_{(-Lys)}$, $G_1+EAA_{(-Thr)}$, and $G_1+EAA_{(-His)}$.

In Experiment II, twenty-four growing rats were randomly assigned to 4 experimental groups (6 rats each) and fed four semi-purified diets: G_1 – 5% of protein, G_1 + the EAA supplement (G_1+EAA) and the variants of the G_1+ the EAA supplement, from which aromatic *i.e.* $G_1+EAA_{(-Phe,\ -Tyr)}$, and branched-chain amino acids, *i.e.* $G_1+EAA_{(-Leu,\ -Ile,\ -Val)}$, were selectively removed. The composition of EAA supplements is given in Table 1.

TABLE 1. Composition of essential amino acids of experimental diets.

Amino acids (EAA)	g/kg diet
Thr	1.41
Val	1.45
Ile	1.33
Leu	2.01
Tyr	1.01
Phe	1.32
His	0.65
Lys	1.75
Cys	0.47
Met	0.31

Nitrogen balance and preparation of biological material for analyses. Nitrogen balance experiments and preparation of biological materials were conducted as described previously [Pysz & Pisulewski, 2004].

Chemical analyses. 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 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

TABLE 2. Effect of the absence of individual amino acids or their groups from the diet with essential amino acid supplements (G_1+EAA) on N-retention.

Diet	N retention (mg/kg $W^{0.75}$)
G_1	84.6 ± 11.3^{ab}
G_1+ EAA	134.9 ± 17.3^{e}
$G_1+EAA_{(-Met)}$	81.4 ± 20.4^{a}
$G_1+EAA_{(-Lys)}$	88.4 ± 12.2^{abc}
$G_1+EAA_{(-Thr)}$	103.8 ± 8.0^{bcd}
$G_1+EAA_{(-Phe.\ -Tyr)}$	106.3 ± 5.3^{cde}
$G_1+EAA_{(-His)}$	117.0 ± 20.1^{de}
$G_1+EAA_{(-Leu.-Ile.\ -Val)}$	131.7 ± 15.5^{e}

Data in the columns marked with different letters differ significantly ($p<0.05$)

As indicated in Table 2, omitting Met from the EAA supplement caused a significant ($p<0.05$) decrease in mean N retention (-53.5 mg/kg $W^{0.75}$) to the level of 81.4±20.4 mg/kg $W^{0.75}$, *i.e.* the level observed in animals fed G_1 diet only (without the EAA supplement). Also, omitting Lys or Thr led to significant ($p<0.05$) decreases in N retention (-46.5 or -31.1 mg/kg $W^{0.75}$) to 88.4±12.2 or 103.8±8.0 mg/kg $W^{0.75}$, respectively. On the other hand, omitting aromatic amino acids (Phe and Tyr) or His caused non-significant ($p>0.05$) decreases in N retention (-28.6 mg/kg $W^{0.75}$ or -17.9 mg/kg $W^{0.75}$) to 106.3±5.3 mg/kg $W^{0.75}$ or to 117.0±20.1 mg/kg $W^{0.75}$, respectively. Similarly, omitting branched-chain amino acids (Leu, Ile and Val) produced negligible, non-significant ($p>0.05$) decrease in N retention (-3.2 mg/kg $W^{0.75}$) to 131.7±15.5 mg/kg $W^{0.75}$.

The obtained results (Table 3) indicate unequivocally that Met, Lys and Thr were the first, second and third limiting amino acids in growing rats fed peas as the only protein source. The data on Phe + Tyr and His are less obvious. Moreover, under the experimental conditions of this study, Leu, Ile and Val do not seem to be limiting amino acids. Also, as shown in Table 3, the amounts of individual EAA (Met + + Cys, Lys, Thr, Tyr + Phe and His), present in the EAA supplement were: 15.4, 35.0, 28.2, 46.7 and 12.9 mg/g of protein, respectively. Similarly, the amounts of branched-chain amino acids (Leu, Ile and Val) were 40.3, 26.7 and 29.0 mg/g of protein, respectively. Furthermore, the extent of deficit of successive limiting amino acids, *i.e.* Met + Cys, Lys and Thr, derived from the values of their utilization in growing rats, was estimated to be: 15.4, 32.2 and 17.5 mg/g of protein, respectively. Deficit of aromatic amino acids Phe + Tyr reached 26.6 mg/g of protein, while the respective value for His was 4.6 mg/g of protein. Consequently, the amounts of individual EAA (Met + Cys, Lys, Thr, Tyr + Phe and His)

TABLE 3. Contents of essential amino acids (in mg/g of protein) in basal diet with EAA and the calculated optimal amino acid composition of model pea protein of cultivar *Cyrcon*.

Amino acids	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Val
Basal diet G_1	19.6	40.4	61.0	53.0	23.4	70.8	42.7	44.0
EAA supplement	**12.9**	**26.7**	**40.3**	**35.0**	**15.4**	**46.7**	**28.2**	**29.0**
G_1+EAA	32.5	67.1	101.3	88.0	38.8	117.5	70.9	73.0
EAA utilization	0.36	0.06	0.06	0.92	1.00	0.57	0.62	0.06
Extent of deficit	**4.6**	**1.6**	**2.4**	**32.2**	**15.4**	**26.6**	**17.5**	**1.7**
Optimal amino acid composition of model pea protein	**24.2**	**42.0**	**63.4**	**85.2**	**38.8**	**97.4**	**60.2**	**45.7**

supplements, required to obtain the maximum efficiency of the model protein utilization, were: 23.4+15.4=38.8, 53.0+32.2=85,2, 42.7+17.5=60.2, 70.8+26.6=97.4 and 19.6+4.6=24.2 mg/g of protein, respectively. The above supplements (mg/g) were calculated using the following formula: basal diet G_1 + (EAA Supplement ∗ EAA utilization); *e.g.* Met+Cys=23.4+(15.4×1.00)=38.8 mg/g. Since under present experimental conditions, branched-chain amino acid (Leu, Ile and Val) supplement has not been utilized, the optimum content of these amino acids can be considered equal to that in the model protein, *i.e.* 63.4, 42.0 and 45.7 mg/g of protein, respectively. In addition, we clearly state that we did not analyse pea protein for its tryptophane content and therefore our calculation did not take into account a possible limiting role of this amino acid.

DISCUSSION

As demonstrated in Part I of this study [Pysz & Pisulewski, 2004], N-retention responses of growing rats to supplementation of pea protein, with either the same protein at a level equivalent to 0.66 of dietary protein, derived as (1-BV)/BV, or the mixture of EAA, formulated to reproduce pea protein, were *equal*. Consequently, the EAA supplement, defined as above, could be used to determine the order and extent of amino acid limitation in pea protein, by an amino acid removal technique. As it was assumed, this technique allowed the identification of methionine, lysine and threonine as the first, second and third limiting amino acid in rats fed peas as the only protein source. Moreover, it identified the extent of their limitation to be: 15.4, 32.2, 17.5 mg/g of protein, respectively. The latter was equal to the minimum amounts of individual EAA required to obtain the maximum efficiency of pea protein utilization (*i.e.* N retention corresponding to BV=1), in growing rats. Similar concepts were successfully applied to determine amino acid requirement in rats [Heger & Frydrych, 1985; Gahl *et al.*, 1991; Benevenga *et al.*, 1994] and pigs [Fuller *et al.*, 1989; Chung & Baker, 1992]. They have also been used for evaluation of amino acid requirements in humans [Kurpad *et al.*, 1998; Millward *et al.*, 2000]. In those studies, the technique of selective removal of EAA involved their partial or total removal from their mixtures (considered initially balanced), which were the only source of amino acids in respective diets. However, in contrast to the above methods, the present approach, by using the formula (1-BV)/BV, may be used to determine the *minimum* supply of all individual EAA, required to optimise the utilization of any food protein, and closely approximates the limiting amino acids of such a protein [Storm & Orskov, 1984].

The derived optimum amino acid pattern of pea protein, when fed to growing rats (Table 3), may be interpreted as the requirement of this species for individual EAA. In such experiments, the optimum amino acid composition of dietary protein is assumed to be closely related to respective composition of tissue proteins in growing animals. More precisely, this requirement should reflect the growth and maintenance needs of a growing rat. Indeed, the latter were found different from the former in rats [Heger & Frydrych, 1985; Benevenga *et al.*, 1994] and pigs [Fuller *et al.*, 1989; Chung & Baker, 1992]. Table 4 shows EAA requirements of growing rats (including semi-essential cystine and tyrosine), determined by us and compared with results of other studies. It can be seen, that amino acid requirements, as derived in the present study, generally agree with literature data and reflect amino acid composition of muscle tissue in growing rats. However, our values for Leu, Met + Cys and Val were lower while those for Lys, Phe + Tyr and Thr were higher than the majority of literature data. These differences result probably from various experimental methods used for evaluation of amino acid requirements in rats. It may be particularly due to mathematical interpretation of curvilinear relationships between amino acid intakes and biological effects [Heger & Frydrych, 1985; Fuller *et al.*, 1989; Fuller & Garthwaite, 1993; Benevenga *et al.*, 1994; Remmenga *et al.*, 1997]. On the other hand, the effect of maintenance requirement on the presented values was negligible as it does not exceed 5 % of a sum of the requirements for growth + maintenance in rats [Benevenga *et al.*, 1994].

The requirements derived for sulphur (Met + Cys) and

TABLE 4. Comparison of amino acid requirements (mg/g of protein) in growing rats and chemical score (CS) obtained in the present study with literature data.

Amino acids	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Trp	Val
Present study (CS)	**24** **0.83**	**42** **0.95**	**63** **0.97**	**85** **0.62**	**39** **0.62**	**97** **0.72**	**60** **0.70**	**-**	**46** **0.98**
NRC [1978]	25 0.80	42 0.95	63 0.97	58 0.91	50 0.48	67 0.72	42 1.00	13	50 0.88
NRC [1995]	19 1.05	41 0.98	71 0.86	61 0.87	65 0.37	68 1.03	41 1.02	13	49 0.90
AIN, Reeves *et al*. [1993]	26 0.77	48 0.83	86 0.71	73 0.73	47 0.51	101 0.69	38 1.10	12	56 0.79
Benevenga [1994]	26 0.77	59 0.68	100 0.61	86 0.62	92 0.26	95 0.74	59 0.71	18	70 0.63
Heger and Frydrych [1985]	30 0.67	49 0.82	79 0.77	75 0.71	62 0.39	86 0.81	53 0.79	15	62 0.71
Composition of tissue proteins in rats Davis *et al.* [1993]	30	39	85	77	-	-	43	-	52

aromatic (Phe + Tyr) amino acids are of particular interest (Table 4). Sulphur amino acid requirement (39 mg/g of protein) was generally lower than that reported in earlier studies. However, it seems that requirements for these amino acids, exceeding 50 mg/g of protein, can be overestimated. The value of 40 mg/g of protein [Peace *et al.*, 1986; Sarwar, 1997] appears to be actual requirement of growing rats, which is comparable with the value obtained in the present study. Contribution of methionine and cystine to a sum of sulphur amino acids was 40% and 60%, respectively. Such ratio generally agrees with requirement of growing rats [Sowers *et al.*, 1972] and pigs [Fuller *et al.*, 1989; Chung & Baker, 1992]. Contribution of Phe and Tyr to relatively high requirement for aromatic amino acids (97 mg/g of protein) amounted to 57% and 43%, respectively. Similar ratio is considered optimal for ideal protein for growing pigs [Fuller *et al.*, 1989; Chung & Baker, 1992].

The values of chemical score (CS) given in Table 4, identified Met + Cys and Lys as the first limiting amino acids, and Phe + Tyr and Thr as successive limiting amino acids in pea protein, when related to the derived optimum amino acid composition of this protein (Table 1). Identification of the above amino acid limitations could be expected as these amino acids have long been regarded as deficient in the model protein. Of these, Met + Cys were limiting at the level of $CS_{Met+Cys}=0.62$. This value was higher than those calculated for other amino acid patterns (Table 4) and could have probably resulted from over-estimation of actual sulphur amino acid requirement (40 mg/g of protein), [Peace *et al.*, 1986; Sarwar, 1997] in other patterns. Validity of our results is further confirmed by numerical agreement of biological value of the model protein of peas (BV=0.6) and CS of this protein ($CS_{Met+Cys}=0.62$); the calculation of the later was based on our optimum amino acid pattern. However, it is not surprising because all indices of biological quality of proteins are a function of the first limiting amino acid [Bender, 1982]. In conclusion, requirements for EAA in growing rats, as determined in the present study (Table 3) can serve as a reliable amino acid pattern to evaluate nutritional value of proteins (in growing rats) by the method of chemical score (CS).

The present approach can be directly applied to evaluate amino acid requirements in growing children and adolescents, and indirectly, to estimate nutritional value of food proteins. It is obvious that this experiment can be used in studies on amino acid requirements in growing humans, being in positive N-balance. A need for such studies and three areas to be re-evaluated have been recently advocated [Jackson, 2001; Millward, 2001; Reeds, 2001]. First, the classic idea of EAA and their physiological requirements have been questioned in view of the findings demonstrating *de novo* synthesis of lysine, an amino acid considered absolutely essential [Millward *et al.*, 2000]. There are also fundamental differences between amino acid requirements in adults, determined on the basis of C and N balance. Apart from technical reasons [Fuller & Garlick, 1994], they can stem from different metabolic pathways of N and C of the tested amino acids [Millward, 1998; Young, 1998]. At the same time, application of the present approach in adults (in zero N-balance) is unlikely. Second, EAA requirements depend on their digestibility and availability. Initial high digestibility and availability of EAA can be significantly altered during their intestinal and hepatic metabolism [Reeds, 2001] and affect the final requirement values. Third, there are serious doubts concerning the validity of current methods of estimation of EAA requirements in human population. Individual variability of human amino acid requirements is difficult to define and thus may affect the variability range [Jackson, 2001; Reeds, 2001].

The present approach can be indirectly applied to re-evaluate the results of the PDCAAS method [FAO/WHO, 1991] and newly proposed methods such as the relative protein value (RPV) method [Young *et al.*, 1989] and the net post-prandial protein utilization (NPPU) method [Tome & Bos, 2000], in which protein quality is invariably determined as a function of the first limiting amino acid. As indicated before, the present approach allows the identification of all EAA in a given protein and also, extent of their limitation. Moreover, by quantitative determination of the extent of EAA deficits, the presented approach makes it possible to precisely assess the sources of dietary proteins which can complement each other in a human diet most effectively.

CONCLUSIONS

1. Selective removal of individual amino acid from the synthetic EAA mixtures, with the same composition as that of pea protein, allowed the identification of Met, Lys and Thr as the first, second and third limiting amino. The extent of their limitation was 15.4, 32.2, 17.5 mg/g of protein, respectively, in rats fed peas as the only protein source.
2. The proposed approach can be used to re-evaluate the results of the PDCAAS [FAO/WHO, 1991], RPV [Young *et al.*, 1989] and NPPU method [Tome & Bos, 2000], in which protein quality is invariably determined as a function of the first limiting amino acid.
3. The proposed approach makes it possible to precisely assess the sources of dietary proteins which can complement each other, in a human diet, most effectively.

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NOWA BIOLOGICZNA METODA OCENY JAKOŚCI BIAŁKA ŻYWNOŚCI. CZ. II. OKREŚLENIE KOLEJNOŚCI AMINOKWASÓW LIMITUJĄCYCH I WIELKOŚCI ICH NIEDOBORU W BIAŁKU GROCHU PRZY UŻYCIU TECHNIKI SELEKTYWNEGO USUWANIA AMINOKWASÓW

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Celem pracy było określenie kolejności aminokwasów limitujących i wielkości ich niedoboru w białku grochu przy użyciu techniki selektywnego usuwania aminokwasów. Podstawowym źródłem białka w dietach półsyntetycznych były nasiona grochu. Białko grochu uzupełniano syntetycznymi aminokwasami niezbędnymi w ilości i składzie podanym w tab.1, a następnie z powyższej mieszaniny selektywnie usuwano pojedynczo aminokwasy Met, Lys, Thr, His, oraz grupami aminokwasy aromatyczne Phe+Tyr, i o rozgałęzionych łańcuchach węglowych Leu, Ile, Val. Doświadczenia żywieniowe przeprowadzono metodą bilansową Thomasa-Mitchella w modyfikacji Egguma [1973] na albinotycznych szczurach laboratoryjnych szczepu Wistar.

Usunięcie Met z mieszaniny aminokwasów niezbędnych uzupełniającej dietę podstawową prowadziło do istotnego ($p<0,05$) zmniejszenia retencji N, podobnie jak w przypadku usunięcia Lys i Thr. Po usunięciu His, a także łącznym Phe+Tyr zmiany retencji N nie były istotne statystycznie ($p>0,05$). Natomiast usunięcie aminokwasów o rozgałęzionych łańcuchach węglowych (Leu, Ile, Val) nie spowodowało zmniejszenia retencji N. Na podstawie powyższych wyników ustalono kolejność aminokwasów limitujących (Met, Lys i Thr) oraz zakres ich niedoboru (Met 15,4; Lys 32,2 i Thr 17,5 mg/g białka) dla białka grochu (z pominięciem tryptofanu) i tym samym zapotrzebowanie aminokwasowe rosnącego szczura.