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INVESTIGATION of RED BEET [*Beta vulgaris* L. (subsp. *rapa f. rubra*)] JUICE PROTEINS for VITAMIN B_{12} BINDING

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SUMMARY

This work describes the spectrophotometric survey of 20-70% saturated ammonium sulphate fraction proteins of red beet juice specific for interaction with vitamin B_{12}. After the addition of extraneous vitamin B_{12}, the maximum absorption appearing at 260 nm for 20% fraction proteins, considerably increased and shifted to 247 nm. The shift in the absorption spectrum was suspected to be due to binding of proteins with vitamin B_{12}. To reveal the interaction of this fraction proteins with vitamin B_{12}, radioisotope dilution, competitive binding assay and ligand blot methods were used. 20% saturated ammonium sulphate fraction proteins were shown to contain endogenous vitamin B_{12} and cobalt (Co^{+2}) and rich in phenylalanine but no specific proteins were elucidated for the labelled vitamin B_{12} binding by competitive binding assay, radioisotope dilution and ligand blot methods. The maximum absorption appearing at 260 nm instead of 280 nm may be due to presence of high phenylalanine and low tyrosine contents not to binding of proteins to vitamin B_{12}.

Key Words: Red beet, vitamin B_{12}, vitamin B_{12} binding proteins

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Ö Z E T

Bu çalışmada %20-70 amonyum sulfat doygunluğunda çöktürülen kırmızı pancar suyunun çözünür proteinlerinin, B_{12} vitamini ile etkileşimleri spektorofotometrik olarak incelenmiştir. Dışarıdan B_{12} vitamini katıldıktan sonra %20'lik amonyum sülfat doygunluğunda çöktürülen proteinleri 260 nm'de maksimum absorpsiyonu anlamlı bir şekilde arttı ve 247 nm'ye kaydı. Bu dalga boyundaki kaymanın B_{12} vitamini ile bağlanmadan ötürü olabileceğinden şüphelenilmiştir. Yarışmalı bağlanma, radyoizotop sulandırma ve ligand blot metodları kullanılarak işaretli B_{12} vitamini ile bu fraksiyondaki proteinler arasında bağlanma olup olmadığı araştırılmıştır. %20 amonyum sülfat doygunluğundaki protein fraksiyonunda B_{12} vitamini, Co^{+2} ve fenilalaninin fazla miktarda bulunduğu ama proteinlerin işaretli B_{12} vitamini ile herhangi bir etkileşim göstermediği saptanmıştır. Maksimum absorbansın 280 nm yerine 260 nm de belirmesi, proteinlerin vitamin B_{12} ile etkileşmesinden değil, bu fraksiyondaki proteinlerin fazla miktarda fenilalanin, az miktarda tirozin içermesinden ötürü olabilir.

INTRODUCTION

Vitamin B_{12}, a relatively large molecule with a molecular weight of 1355, is one of the vitamins synthesized only by microorganisms (Murray *et al,* 1993; Battersby, 1993; Bicker *et al,* 1994), although some reports claim that plants synthesize detectable amounts of vitamin B_{12}, provided that animal manure is used (Farquharson *et al.,* 1977; Lontoc *et al.,* 1968; Mozafar *et al.,*1992). The presence of vitamin B_{12} at relatively high concentrations in red beet juice [*Beta vulgaris* L. (subsp. *rapa f. rubra*)] has been established by radio immunoassay (Enginün *et al.,* 1995) and microbiological assay utilizing *Lactobacillus leichmannii* (ATCC 7830) (Ogan *et al.,* 2000), implying the presence of vitamin B_{12} binding proteins.

Spectrophotometric scanning may provide a fast and convenient method for determining the possible presence of such proteins before further attempting to purify them. In this study the direct absorption spectra of 20% saturated ammonium sulphate fraction proteins and of the proteins plus vitamin B_{12} against solvents as blank, and of proteins after incubation with vitamin B_{12} and vitamin B_{12} after incubation with the proteins as difference spectra against the relevant blanks containing only vitamin B_{12} and only protein were then recorded at 190-400 nm. and compared to the absorption spectrum of bovine serum albumin (BSA) with vitamin B_{12}. The spectrophotometric

survey of 20% saturated ammonium sulphate fraction proteins suggested a further attempt to study this fraction. Not only the spectrophotometric evidence to rest on absorption peak, the presence of endogenous cobalt (Co^{2+}) and vitamin B_{12} was confirmed by quantitative assays and the amino acid composition of the precipitated protein fraction was determined. Specificity of those proteins for ^{57}Co labelled cyanocobalamine (^{57}Co CN-Cbl) were elucidated by the competitive binding assay, radioisotope dilution (RID) and ligand blot methods.

RESULTS AND DISCUSSION

The presence of vitamin B_{12} in red beet juice [*Beta vulgaris* L. (subsp. *rapa f. rubra*)] indicates red beet might have developed elaborate mechanisms for the absorption, transport and main-tenance for this trace element (Fries, 1962 and Muhammad *et al.*,1993). Spectrophotometric scanning may provide a fast and convenient method for determining the possible presence of such proteins before further attempting to purify them.

Table 1. Vitamin B_{12} and cobalt content of 20% saturated ammonium sulphate fraction.

Sample	Vitamin B_{12} M	Cobalt (ppb)
20% Fraction	$1.24X10^{-8}$	0.71

Table 2. The amino acid composition of 20% saturated ammonium sulphate fraction proteins as mg/L.

Amino acid	mg/L
Alanine	9.1
Arginine	5.6
Aspartic acid	3.7
Glutamic acid	7.2
Glycine	5.8
Histidine	5.5
Isoleucine	33.8
Leucine	38.4
Lysine	25.7
Methionine	5.5
Phenylalanine	11.0
Serine	9.6
Threonine	7.4
Tyrosine	< 1.0
Valine	17. 1

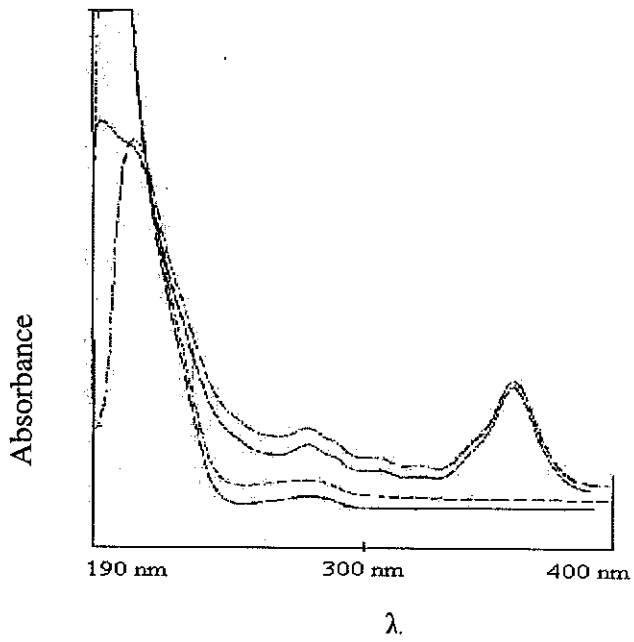

Fig. 1. The absorption spectrum of vitamin B_{12} (10 μg/mL) after incubation with the 20% saturated ammonium sulphate fraction proteins (50 μg/mL) as difference spectra against the relevant blanks;
——— Vit B_{12} in phosphate buffer/ phosphate buffer,
—·—·— Proteins in phosphate buffer + vit B_{12} in phosphate buffer/ Proteins in phosphate buffer,
– – – Proteins in phosphate buffer/phosphate buffer,
····· Proteins in phosphate buffer+ vit B_{12} in phosphate buffer/vit B_{12} in phosphate buffer,

The spectrophotometric survey of each 20-70% saturated ammonium sulphate fraction soluble proteins revealed that the 20% saturated ammonium sulphate fraction proteins considerably interacted with the addition of extraneous vitamin B_{12}. As shown in Figure 1, the optical density of this fraction proteins displayed a maximum absorption at 260 nm instead of 280 nm. This maximum absorption peak shifted to 247 nm and considerably increased after the addition of vitamin B_{12} and gave even a shoulder between 300-350 nm implying that one or more proteins were already complexed with vitamin B_{12}. The absorption spectrum of vitamin B_{12} after incubation with the proteins as difference spectra against the relevant blanks; containing respectively only vitamin B_{12} and only proteins displayed three peaks near 225 nm, 245 nm and 380 nm and there was also a slight increase in the absorption maximum at 380 nm. The BSA which does

not bind vitamin B_{12} was used to demonstrate that the optical densities of BSA and vitamin are additive and there is no shift in the absorption maximum wavelength at 280 nm (Figure 2). In addition to the spectrophotometric evidence Co^{2+} and vitamin B_{12} were quantitated (Table 1), and the amino acid composition of the fraction proteins was also determined (Table 2). Vitamin B_{12} and Co^{2+} contents enhance the presence of potential vitamin B_{12} specific proteins in this fraction. Amino acids with aliphatic side chains constitute the greatest portion. The assignment of possible vitamin B_{12} specific proteins at 260 nm may be due to high phenylalanine but very low tyrosine content of the proteins (Mathews *et al.*, 1990).

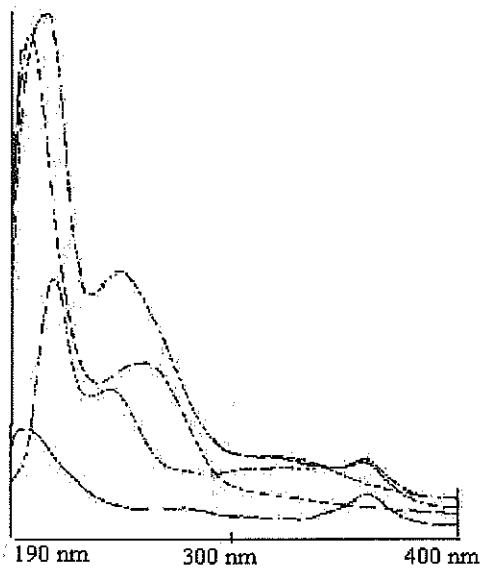

Fig. 2. The absorption spectrum of vitamin B_{12} (10 μg/mL) after the incubation with BSA (50 μg/mL) as difference spectra against the relevant blanks;
_____ BSA in phosphate buffer / phosphate buffer (bottom)
- - - - BSAin phosphate buffer+vit.B_{12} in phosphate buffer/ vit. B_{12} in phosphate buffer,
Vit.B_{12} in phosphate buffer/ phosphate buffer,
BSA in phoshate buffer+vit.B_{12} in phosphate buffer/BSA in phosphate buffer

No binding activity for ^{57}Co CN-Cbl was observed by the RID and competitive binding assay, since the counts as counts per minute for total binding tubes and nonspecific binding tubes were nearly the same (Table 3 and 4). The shift in the

absorption maximum from 280 nm to 260 nm might be due to low tyrosine content of the proteins, not due to a binding activity.

Polyacrylamide gel electrophoresis (PAGE) for the ligand blotting was performed under non-reducing conditions, since even low concentrations of reducing agents (0. 1 %β-mercaptoethanol) cause changes in the binding proteins rendering them incapable, after transfer, of binding to the labeled cyanocobalamin (Hossenlopp *et al.*, 1986). No image was observed on the exposure film after the development of the film.

Red beet juice is a beneficial nutrient supplement since it is associated with human blood and blood forming qualities [Enginün *et al.*, (1995), Krantz *et al.*, (1980) and is a good source of essential amino acids] (Table 2).

Table 3. Mean counts/minute obtained for radio isotope dilution method

	Mean counts/minute
NSB (non specific binding)	127
1:1 (dilution)	144
1: 4 "	122
4:1 "	98

Table 4. Mean counts/minute for competitive binding method with vitamin B_{12} standards

	Mean counts/minute
TB (total binding)	80
NSB (non specific binding)	83
St 1	85
St 2	86
St 3	89
St 4	78
St 5	76
St 6	72
St 7	69
St 8	70

EXPERIMENTAL

Materials. Red beet *(Beta vulgaris* L. (subsp. *rapa f. rubra*)) was purchased from a wholesaler in Istanbul, Turkey. The roots of red beet approximately 10 kg were thoroughly washed and their juice was squeezed out by a juice extractor and then filtered (approx. 2L).

Vitamin B_{12} was a gift of Roche Co., Istanbul-Turkey, Bovine serum albumin (BSA) was obtained from Sigma Chemicals Co. The rest of the chemicals were purchased from Merck Co. and were all reagent grade.

Precipitation of red beet proteins and spectral analysis: Precipitate of proteins of red beet proteins were collected at 20-70% of ammonium sulphate saturation with 5% increments, desalted and freeze dried (Clark *et al.,* 1977). Each fraction was dissolved in phosphate buffer, pH: 6.4, and adjusted to give a volume containing 50 μg/mL protein by the Lowry Method (Lowry *et al.,*1951). Direct spectra of each protein and the proteins after incubation with vitamin B_{12} (10 μg/mL) as difference spectra against the relevant blanks containing respectively only vitamin B_{12} and only protein were then recorded at 190-400 nm with a Shimadzu UV-visible spectrophotometer (UV-240) (Boyer, 1991). This analysis was repeated several times to check the reproducibility of the results. BSA which does not bind vitamin B_{12} was used to demonstrate that the optical density of BSA and vitamin B_{12} were additive. BSA (50 μg/mL) was incubated with vitamin B_{12} (10 μg/mL) and the spectral analysis was performed as described for fraction proteins

Quantitative vitamin B_{12}, cobalt and amino acid analysis of 20% saturated ammonium sulphate fraction proteins: The final amount of endogenous vitamin B_{12} was determined by microbiological assay utilizing *Lactobacillus leichmannii* (ATCC 7830) (Adjalla, 1993) and Co^{2+} by ICP-MS Hewlett Packard 4500 series (Kishi *et al.,* 1995). Total amino acid composition of the hydrolyzed proteins was retrieved in a private clinical laboratory (Gen Lab İstanbul).

Studies on the binding of vitamin B_{12} by means of the radioisotope dilution technique. 0.5 mL of the assay mixture; contained 10mM phosphate buffer, pH: 6.4, ^{57}Co CN-Cbl, (specific activity: 2 μCi) and the fraction proteins (0.15 mg/ml protein). The mixture was incubated for 2 minutes at 25°C in the dark, immediately filtered through a nitrocellulose acetate membrane filter (0.45μm, 13 mm) and washed three times with 5 ml of 10 mM phosphate buffer, pH: 6.4, containing 0.1 M sodium chloride. Radioactivity on the dried membrane filter was counted (Watanabe *et al.,* 1993).

Competitive binding assays: The study was undertaken to determine the relative binding affinity of ^{57}Co CN-Cbl for the possible specific proteins. A series of 10 mm x 75 mm polypropylene tubes were labelled, then 0.05, 0.10, 0.15, 0.20, 0.25, 0.50, 0.75, 1.00 ml of aqueous solutions of 310 pM CN-Cbl were added to the tubes in triplicate. The volume of the solutions in the tubes was then adjusted to 1. 0 ml with distilled water. The non -specific binding (NSB) and total binding (TB) tubes had distilled water (1.0 ml) added to them. To each of the above tubes, ^{57}Co CN-Cbl (50 µl) was added. The tubes were thoroughly mixed and then incubated for 30 minutes at room temperature. After the incubation, the tubes were centrifuged at 3. 000x g for 30 minutes at 20°C. The supernatants were decanted and the residues were retained for counting in a gamma counter for 1 minute (Fedesov *et al.,* 1995).

Binding proteins by ligand blotting: Polyacrylamide gel electrophoresis (PAGE) was performed (Laemli, 1970) using 10 % homogenous acrylamide slab gels (10x10x0.1) under non-reducing conditions. After the electrophoresis, the gel was soaked in borate buffer, pH: 9.0, for 5 minutes and placed on a nitrocellulose sheet containing 20 % methanol. Electro-blotting was performed under a constant current, 0. 8 A at 15°C for 2 hours (Hossenlopp *et al.,* 1986). After the transfer, the membrane was dried at 37°C for 5 minutes. Thereafter it was soaked at 4°C first, for 30 minutes in saline (0.15 M NaCl, 0.001 M Tris-HCl pH: 7.4), then in saline supplemented with nonylphenoxy polyethoxy ethanol 40 (NP40). The nitrocellulose membrane was then incubated overnight at 4°C with ^{57}Co labeled CN-Cbl in a sealed plastic bag for 15 minutes in saline containing 0.1 % Tween 80 and then washed three times for 15 minutes in saline. The blot was dried and then exposed at –70°C to x-ray film for ~4 days (Towbin *et al.,* 1979).

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